REACTIVE OXYGEN SPECIES-MEDIATED REGULATION OF THE \( \text{Na}^+ / \text{H}^+ \) EXCHANGER, NHE-1 GENE EXPRESSION: A NEW MECHANISM FOR TUMOR CELLS’ RESISTANCE TO APOPTOTIC CELL DEATH

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Abstract

Reactive Oxygen Species have long been known to cause cellular stress and damage. But recently, ROS have been implicated as signaling molecules. Tumor cells display an altered redox status. Our lab has recently shown that expression of a constitutively active form of Rac1 (RacV12) inhibits tumor cell death by apoptosis through intracellular production of superoxide anion (O$_2^-$) (Pervaiz et al. 2001, Oncogene).

Another characteristic of transformed cells is a shift towards alkaline intracellular pH. NHE-1, one of the major pHi regulators, has been shown to be of particular importance in tumor cells. Current study was designed to study the effect of ROS on NHE1 regulation and the role it may play in modulating apoptosis. Our data shows that production of intracellular O$_2^-$ induces transcription of NHE-1 while increase in H$_2$O$_2$ inhibited it. Using Rac mutants, which have differential ability to produce O$_2^-$ in the cell, and drugs that affect the intracellular ROS levels, we were able to show that NHE1 gene is redox-responsive. Changes in NHE1 gene expression were translated into NHE1 protein expression. By over-expressing or silencing NHE-1 gene we show that cell response to apoptotic triggers such as staurosporin and etoposide correlates with the amount of NHE-1 protein expression on the cell surface. Moreover, down-regulation of NHE-1 gene expression in tumor cell lines tested reverted their resistant phenotype. These results support a critical role for NHE-1 expression in tumor cells’ response to anticancer therapy and provide a possible mechanism for Rac1-mediated survival in tumor cells.

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<td>BCECF</td>
<td>2', 7'-bis (2-carboxyethyl)-5, 6-carboxyfluorescein</td>
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<td>DDC</td>
<td>Diethyldithiocarbamate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DPI</td>
<td>Diphenylene iodonium</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>Eto</td>
<td>Etoposide</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
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<td>O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Superoxide dismutase</td>
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<tr>
<td>Sts</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>Tiron</td>
<td>4, 5-dihydro-1, 3 benzene disulfonic acid</td>
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Chapter I

Introduction

I.1. Cell Death

Cell number in a multi-cellular organism is constant but dynamic. Cells are constantly undergoing growth; dead cells are replaced by new ones. Cell death can occur either accidentally or in a pre-determined fashion. Accidental cell death takes place when cells are suddenly exposed to conditions which are incompatible with life, for example, sheer physical stress, chemical poisons, radiation, etc. A process of cell death called “Necrosis” ensues, which leads to disintegration of cellular organelles, cytoplasmic swelling and finally membrane rupturing. On the other hand, cells can also decide to die. This happens when a cell becomes functionally redundant or is no longer needed for the organism. This type of cell death is called “Apoptosis” and comprises of a complex but very well orchestrated chain of events. In physiological circumstances apoptosis is the favorable mode of death as it does not lead to a spillage of intracellular contents into the extra-cellular space, and no or little immune reaction (Steller H, 1995; Wyllie AH et al., 1980). Salient differences between Apoptosis and Necrosis are tabulated in Fig A.

One of the hallmarks of tumor development and maintenance is defiance of tumor cells to execute death signals (Thompson CB, 1995). Thus, a combination of increased proliferation and lack of cell death leads to the development of cancer mass. Cell death could occur via different mechanisms depending upon various factors like initiating triggers, tissue and cell type involved and so on.
I.1.a. Types of Cell Death

Apoptosis and necrosis have classically been defined as two entirely different types of cell death, starting from the factors that can induce cell death, the signaling pathways, death execution and the way body clears away dead cells (Zhaoyu J and Wafik SD, 2005). Despite these differences, recent observations have suggested that there might be some overlapping between these two morphologically distinct types of cell death (Nicotera P and Melino G, 2004; Lockshin RA and Zakeri Z, 2004).

In addition, programmed cell death can occur without the classic morphological features of apoptosis. Historically speaking differentiation between apoptosis and necrosis were based upon morphological features of the dying cells. With in depth studies into the biochemical events occurring during cell death, many different types of cell deaths have now been defined (Melino G et al, 2005; Kroemer G et al, 2005; Kondo Y et al, 2005). Few examples of these other forms of cell death include autophagy, paraptosis, anoikis, Wallerian degeneration and cornification. Except for necrosis, all other forms of cell death are believed to have genetic component (Kroemer G et al, 2005). The type of cell death a particular cell chooses may vary according to the prevailing circumstances.

I.1.b. Programmed Cell Death or Apoptosis

Programmed Cell Death and its morphologic manifestation of Apoptosis is a distinct genetically controlled process. The execution of apoptosis is characterized by a chain of both morphological and biochemical events. These include mitochondrial depolarization, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and formation of membrane bound vesicles termed as apoptotic bodies (Kerr et al., 1972). Apoptosis has proven to be tightly regulated and interwoven with
### Necrosis vs. Apoptosis

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<tr>
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<th>Apoptosis</th>
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<tr>
<td>Genetic Program</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell Membrane</td>
<td>Lysed</td>
<td>Intact, PS Exposure</td>
</tr>
<tr>
<td>Organelles</td>
<td>Lysed</td>
<td>Intact</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Ruptured</td>
<td>Intact</td>
</tr>
<tr>
<td>Nucleus</td>
<td>-</td>
<td>Chr. Cond., DNA Frag.</td>
</tr>
<tr>
<td>Enzymes</td>
<td>None</td>
<td>Caspases</td>
</tr>
<tr>
<td>Receptors</td>
<td>-</td>
<td>Death Receptors</td>
</tr>
<tr>
<td>Regulators</td>
<td>-</td>
<td>Bcl family, IAP</td>
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**Fig. A. Major differences between Apoptotic and Necrotic types of Cell Death.**

PS, Phosphatidylserine; Chr. Cond., Chromatin Condensation; IAP, Inhibitory Apoptotic Protein.
other essential cellular functions. Some of the molecular components of apoptotic machinery have been conserved through evolution (Steller H, 1995). An intact death pathway is required for successful organogenesis in embryonic life and maintenance of normal tissue homeostasis in adult organisms. As opposed to necrosis, apoptosis minimizes the leakage of cell contents into extracellular space, which in turn results in a minimal inflammatory response and tissue damage. Apoptosis has been studied most extensively in the worm, *C elegans*. Genetic studies have identified 14 genes in *C. elegans* that affect programmed cell death (Steller H, 1995), homologues of some of these genes have been identified in mammals. For example, two of *C. elegans’* genes, *ced-9* and *ced-3* (*ced* stands for cell death defective), are homologous to mammalian genes: the proto-oncogene *bcl-2* and *ice* (interleukin-1-β-converting enzyme), respectively.

Deregulation of apoptosis can be very detrimental to the organism. Excessive cell death can lead to a number of diseases, for example, AIDS, neurodegenerative disorders and ischemic injury (Thompson CB, 1995). In contrast, impaired apoptosis is a significant factor in the etiology of diseases like cancer, autoimmune disorders and viral infections.

**I.1.c. Apoptotic Machinery**

Apoptosis is a complex phenomenon of morphological and biochemical processes. The field of apoptosis has witnessed an explosion of information over the past two decades. The *C. elegans* hermaphrodite undergoes a distinct programmed cell death pattern in which the same 131 cells out of 1090 cells die during the development of this worm (Brenner et al., 1974; Sulston et al., 1976). In more complex organisms,
like mammals the regulation of apoptosis and its mechanism is far more intricate and complex.

Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by specific morphological changes in cell structure. Classically, Apoptosis can be initiated with or without the involvement of mitochondria. In cell-surface receptor induced apoptosis, activation of Fas or TNF receptor leads to the activation of initiator caspase 8, followed by the activation of downstream effector caspases (Fig B). In mitochondrial or intrinsic pathway, upon apoptotic triggers there is a release of mitochondrial contents, most notably cytochrome C, into the cytosol. This leads to the formation of a complex between cytochrome C, Apaf1 and pro-Caspase 9, known as “Apoptosome”. Bcl-2 and Bcl-xL block death by preventing the release of mitochondrial contents into cytosol. On the other hand, pro-apoptotic members of Bcl-2 family like Bad and Bax, play an important role in facilitating apoptosis (Fig C).

Executioners of apoptosis include a cascade of proteases termed caspases. Currently 11 human caspases has been identified. Initiator caspases including caspase-1, 2, 4, 5, 9, 11 and 12 interact with upstream adapter molecules and once activated lead to downstream activation of executioner caspases (caspase-3, 6 and 7). A striking feature of these enzymes is their specificity of substrate cleavage after an Asp residue (Degterev A et al, 2003). Caspase activation leads to the cleavage/degradation of a number of cellular proteins like PARP, Lamin A.

To make the picture more complex, other families of proteins have been identified recently which are involved in the regulation of apoptosis. IAPs (Inhibitor of apoptosis proteins) bind to caspases and inhibit their activity. Another player with the
Fig. B. Death-Receptor mediated Apoptosis.

Fig. C. Mitochondrial pathway of Apoptosis.
dual name of Smac/DIABLO has been identified which promotes caspase activation and inhibits xIAP (Douglas RG, 2000). In summary, apoptotic machinery consists of a host of proteins interacting with each other in a complex and intricate manner, one group of proteins favoring apoptosis and the other opposing it. Thus, the decision to die is a matter of balance amongst anti- and pro-apoptotic proteins.

To our interest, numerous studies have demonstrated redox-regulated functional modifications of many of the proteins involved in apoptotic machinery (Dechao L et al. 2004; John JH, 2004; Irani K and Pascal JGC, 1998).

I.2. Reactive Oxygen Species and Apoptosis

Cells generate reactive oxygen species (ROS) during aerobic metabolism. Higher levels of ROS are detrimental to cell’s functions, thus each cell has an extensive antioxidant defense system to scavenge excessive amounts of ROS. The intracellular redox state is controlled by the thioredoxin and glutathione systems (Mates JM and Sanchez-Jimenez F, 1999). Mitochondria are the major source of ROS, where electrons carried by the electron transport chain may leak out of the pathway and react with oxygen to form superoxide (O$_2^-$). Other sources of O$_2^-$ include enzymes such as NADPH oxidase (NOX), lipoxygenases, cyclooxygenases, xanthine oxidase, and cytochrome P450. Once O$_2^-$ is generated it is rapidly converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase. Hydrogen peroxide can then react with Fe$^{2+}$ to form hydroxyl radicals via the Fenton reaction.

I.2.a. Pro and Anti-Apoptotic functions of ROS

Over the past decade or so there has been a paradigm shift in the understanding of ROS and the functions they can play in the cell. The intracellular concentration of
these reactive molecules is kept under tight regulation by cells’ anti-oxidant systems. The anti-oxidant defense mechanisms include scavenger enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (Halliwell B and Gutteridge JMC, 1999). Therefore, accumulation of ROS in the cell is a function of the overall production and the efficiency of the anti-oxidant defences which could be cell specific. Several reports have suggested that phorbol esters stimulate the production of $O_2^-$ not only in phagocytic cells but in other cultured cells as well (Bonser et al, 1986; Fischer et al, 1986). This small amount of $O_2^-$ produced in non-phagocytic cells in response to mitogenic stimuli may play some physiological role in the signal transduction. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) can affect a wide variety of cellular functions (Droge W, 2001; John JH, 2004; Finkel T, 2003). There is a growing consensus that redox status of a cell plays a regulatory role on a wide range of cellular functions: gene transcription, cell proliferation, differentiation and adaptation on one end, and apoptosis and necrosis on the other end of the spectrum (see Fig. D).

Whereas the role of ROS in inducing necrotic cell death is well established, the role of ROS in apoptosis is more controversial. However, there is increasing body of evidence to support the role of ROS in apoptosis.

The activity of caspase proteases has been shown to be influenced by the redox status of these enzymes (Hampton MB et al, 1998). Apoptosis in neutrophils and their clearance by macrophages has also been shown to be ROS dependent (Fadeel B and Kagan VE, 2003).

Fas receptor activation is a major trigger for apoptosis, and it has been shown that $O_2^-$ can act as a natural inhibitor of Fas-induced cell death in tumor cells (Clement MV
Fig. D. Wide array of functions attributed to Reactive Oxygen Species (ROS).

and Stamenkovic I, 1996). Mitochondria can play an important role in modulating apoptosis through generation of ROS. It has been postulated that reversal of mitochondrial $F_0F_1$-ATPase in the inner membrane would lead to an increased concentration of $H_2O_2$ in the cytosol which in turn, would lead to PARP activation and ATP depletion. This depletion in ATP levels could lead to increase acid load in the cell either by production of $H^+$ or inhibition of $H^+$ transporters (Gossmann DL et al, 2004).

Interestingly, cell surface receptor and mitochondrial pathways cross talk with each other through Bid that is a pro-apoptotic member of Bcl-2 family. Pro- and anti-apoptotic proteins of this group form heterodimers and block each other’s activity. Expression levels of these proteins can be controlled at multiple levels: transcription, heterodimer formation and ubiquitination. Anti-apoptotic Bcl-2 family proteins, when phosphorylated, fail to bind to each other. Thus, it has been suggested that the phosphorylation status of Bcl-2 family proteins might affect their ability to regulate apoptosis (Ruvolo PP et al, 2001). ROS have been shown to induce apoptosis by regulating the phosphorylation and ubiquitination of Bcl-2 family proteins (Dechao L et al, 2004).

The tumor suppressor p53, nicknamed Guardian of the Genome, plays an important role in the regulation of cellular response to DNA damage. p53 has been shown to participate in sensing oxidative DNA damage and modulates BER (base excision repair) function in response to persistent ROS stress (Achanta G and Huang P, 2004).

In a recent study, stress-induced p53 activation showed strong ROS sensitivity both in leukemic and normal lymphocytes. These observations identified mitochondrial activity and ROS levels, as a critical intracellular determinant of the p53 stress
sensitivity and suggest potential implications of this linkage in the mechanisms of chemoresistance of acute leukemia cells (Karawajew L et al, 2005).

Thus, it can be concluded that ROS can modulate or alter the activity of a number of very important proteins involved in cell death. Process of apoptosis can be divided into three distinct phases: initiation, effector and degradation. ROS can be involved in all three of these phases.

Reactive Nitrogen Species (RNS) have a more established role in modulation of cell death. Nitric oxide (NO) is an important bioregulatory molecule in the nervous, immune and cardiovascular systems. NO participates in the regulation of many cellular functions as well as in cytotoxic events. It possesses a controversial effect on cell viability by acting both as a protection against apoptotic stimuli or by inducing apoptosis when produced at elevated concentrations (Blaise GA et al, 2005).

The role of ROS will be discussed in more detail as the work presented in this manuscript was undertaken to study the role of ROS in apoptosis.

I.2.b. Ras and Superoxide anion production

Rac1 is a ubiquitously expressed small GTP-binding protein, that functions downstream of oncogene Ras. p21\textsuperscript{ras} (c-Ras) has many functions in the cell, including proliferation, differentiation, apoptosis and cytoskeletal organization. Mutations in a ras allele that make it constitutively active have been found in 30% of all human tumors, making it the most widely mutated human proto-oncogene. Multiple pathways exist downstream of Ras, including activation of Rac1.

Activated Rac1 leads to the generation of ROS, including O$_2^-$ (Irani K and Pascal JGC, 1998). Activation of Rac is classically known to trigger clustering of an enzyme complex, NADPH oxidase (NOX) in phagocytic cells. Activation of NADPH in these
cells catalyses the generation of $O_2^-$ (also known as “respiratory burst”) which in turn kills the ingested bacteria. Until recently, the single example of ‘deliberate’ generation of ROS in mammalian cells was the NOX of phagocytes (Phox). This enzyme is inactive in resting neutrophils, but is activated by exposure to microorganisms or inflammatory mediators, resulting in the robust production of ROS. Although the exact structure and localization of NADPH-like enzyme system has not been identified in non-phagocytic cells, Mox1 (mitogenic oxidase 1) has been cloned and characterized as a homologue to neutrophil gp91phox, which participates in ROS production (Suh YA, 1999). In contrast to a robust production of ROS in phagocytic cells, lower levels of ROS produced in non-phagocytic cells appear to act as secondary messengers or signaling molecules. Recent data suggests ROS produced downstream of Rac might play a role in the regulation of growth, transformation and apoptosis (Finkel T et al, 1999; Irani K et al, 1997). Rac isoform 1 has been identified in many non-phagocytic cells and is responsible for production of intracellular $O_2^-$, as opposed to isoform 2 that has been described in phagocytic cells. The expression of these enzymes in various tissues provides evidence that generation of ROS is a general feature of many and perhaps all cells. Many cell types express NOX enzymes, probably accounting for the diverse cellular ROS generation seen in many of the earlier studies. Examples of non-phagocytic cells where NOX enzymes or its components have been identified include osteoclasts, fibroblasts, glomerular mesangial cells, chondrocytes, endothelial cells and keratinocytes (Bunn and Poyton, 1996; Suh YA, 1999).

As described earlier, $O_2^-$ is the primary ROS generated by normal cellular metabolism, whereas $H_2O_2$ is a catalytically derived intermediate in the conversion of $O_2^-$ to $O_2$ (Fridovich I, 1976; Halliwell B and Gutteridge JMC, 1989). In phagocytic...
cells, large scale production of Nitric Oxide (NO) by macrophages, or $O_2^-$ by neutrophils, provides the host with defense function against invading pathogens, while when produced in smaller amounts in non-phagocytic cells, these same reactive molecules instead of causing damage to the cell function as signaling molecules (Finkel T, 2001).

I.2.c. Superoxide anion and inhibition of apoptosis

Mammalian cells possess multiple sources of ROS generation; most evidence suggests that plasma-membrane associated oxidases may provide one source of ROS associated with resistance to apoptotic triggers. $O_2^-$ has been shown to contribute to the unchecked proliferation in Ras-transformed fibroblasts (Irani K and Pascal JGC, 1998). Although the exact source of $O_2^-$ in non-phagocytic cells is still under investigation, Rac has been implicated as a major component of $O_2^-$-generating system, and presence of NOX enzymes in a variety of cells suggests a role for ROS in various cellular functions.

In addition, plasma membrane of many cells has another ROS-generating enzyme utilizing NADH as an electron donor. Both of these flavin-containing oxidases are inhibited by diphenylene iodonium (DPI). Taken together, NADH and NADPH oxidases may provide candidate sources of $O_2^-$ production associated with cells’ resistance to apoptotic cell death. As described in the previous section, $O_2^-$ can block the Fas-induced cell death in tumor cells.

The regulation of tumor cells’ sensitivity to death stimuli has been shown to be linked to the intracellular levels of $O_2^-$ and $H_2O_2$ (Pervaiz S and Clement MV, 2002 and 2004; Clement MV and Pervaiz S, 1999 and 2001). Interestingly, an increase in intracellular $O_2^-$ concentration achieved by either its direct overproduction (Clement
MV and Stamenkovic I, 1996), drug-induced (Pervaiz S et al, 1999; Ahmad KA et al, 2004), activation of the small GTPase Rac1 (Pervaiz S et al, 2001), or as a result of an inhibition of the O$_2^-$ scavenger Cu/Zn SOD (Pervaiz S et al, 1999), inhibits tumor cell apoptosis triggered by either the CD95 receptor or anticancer drugs. In contrast, H$_2$O$_2$ is a widely accepted trigger of apoptotic cell death (Hirpara JL et al, 2001) and non-toxic levels of H$_2$O$_2$ sensitize cells to death triggers (Clement MV et al., 1998).

Earlier reports have highlighted the regulatory role of intracellular redox status on death signaling by demonstrating an effect on caspase family protease, the central executioners of apoptotic signals (Hampton M and Orrenius S, 1998; Chandra J et al, 2000).

I.3. Intracellular milieu

Various mechanisms of how ROS can lead to cell transformation have been proposed. O$_2^-$ or other “oxidants” may induce targeted damage to chromosomal DNA, leading to enhanced rate of oncogenic mutations or they can directly regulate the signaling cascade that underlies malignant transformation (Irani K et al, 1997). ROS have been shown to activate NF-κB, a transcription factor whose activation has been linked to apoptosis. Evidence has grown in this poorly understood field and many signal-transducing proteins and transcription factors have been added to the list of “redox-sensitive” proteins (Sundaresan M et al, 1996).

Apoptosis is a tightly regulated chain of reactions that involves many enzymatic reactions and proper functioning of all of its components is essential to execute a cell in a predetermined fashion. The executioners of apoptosis, especially caspases are very sensitive to redox alterations and require a reducing environment to be functional. All caspases contain an active site thiol group necessary to perform their
Fig. E. A model of ROS-mediated regulation of Apoptosis.

Figure adapted from Pervaiz S and Clement MV (2002a).
Receptor (CD95/Fas)-induced apoptosis is mediated by early caspase 8 recruitment and activation, which can induce a drop in cytosolic pH (pHc) and facilitate the activation of downstream executioner caspases, such as caspase 3. Other stimuli, such as anticancer drugs and ultraviolet irradiation (UV) are dependent upon mitochondrial death factors for efficient execution. One scenario could be a direct stimulation of mitochondrial ROS production, which leads to H$_2$O$_2$-mediated membrane damage and escape of proapoptotic factors like cyt c. H$_2$O$_2$ can also diffuse out from the mitochondria and trigger cytosolic acidification, thereby creating a permissive environment for caspase activation. The inhibitory effect of O$_2^-$ (NADPH oxidase- or mitochondria-derived) on the apoptotic pathway could be via blocking upstream or downstream caspase activation directly or by inhibiting cytosolic acidification.
function (Thornberry NA and Lazebnik Y, 1998). This thiol group renders them particularly susceptible to redox modification by oxidation. Such chemical modification results in loss of their catalytic activities. In addition treatment of cells with exogenous oxidizing agents triggers apoptosis in a variety of cell types (Hampton M and Orrenius S, 1998).

Recent findings in our lab have led us to believe that the delicate balance of ROS production and elimination leads to an intracellular environment that may be favorable to apoptotic pathway on one end or it may be non-conducive on the other. To this end, the most interesting observations have been made with regards to a complex interplay between different ROS. Balance between the intracellular level of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) seems to play a critical role in determining whether a cell is ready to die or not (Clement MV and Pervaiz S, 2001). As discussed in the previous section, hydrogen peroxide at non-toxic conditions leads to apoptotic cell death. Surprisingly, this effect of \( \text{H}_2\text{O}_2 \) seems to be a function of reduced levels of \( \text{O}_2^- \) anion in the cell (Clement MV et al, 1998). The mechanism of this relationship is not completely understood, and is currently a main focus of research in our lab.

I.3.a. Intracellular pH (pHi)

Interestingly, \( \text{O}_2^- \) has been suggested to affect intracellular pH (pHi) (Shibanuma M et al, 1988). Recent data from our group also suggested that redox regulation of cell survival in tumor cells could be associated with regulation of intracellular pH (Pervaiz S and Clement MV, 2002a) (see Fig B).

Tumors, although may arise from many different genetic alterations, lead to a loss of normal growth control mechanisms. Two phenotypes common to all tumor cells are cellular alkalinization and a shift towards glycolytic metabolism. Changes in
intracellular pH (pHi) can affect many cellular functions including metabolism, cell growth and cell mobility, and so on. Cell metabolism can be affected by pH-sensitive metabolic enzymes, such as phosphofructokinase. Changes in pHi have also been shown to affect polymerization of cytoskeletal elements like tubulin and actin. In tumor cells the pHi is more alkaline as compared to their normal counterparts.

I.3.b. pHi and Apoptosis

The relationship between pHi and extracellular pH (pHe) within a tumor have long been a highly controversial issue. However, in past few years it has been repeatedly shown that cancer cells have intracellular alkalinity (Harguindeguy S et al, 2005). A number of studies have highlighted the key role of increased pHi in tumor transformation, cell survival and metastatic potential of a tumor (Reshkin SJ et al, 2000; DiGiammarino J et al, 2002; Pouyssegur J et al, 2001; Cardone RA et al, 2005) A direct cause and effect relationship among the degree of Multiple Drug Resistance (MDR) and the elevation of tumor pHi has been recognized by many different groups that have studied the dynamic interrelationships between cell pHi and MDR (Keizer HG and Joenje H, 1989; Weinsburg JH et al, 1999).

Cells undergoing apoptosis show intracellular acidification. A wide range of apoptotic triggers lead to this intracellular acidification including UV irradiation, staurosporine, etoposide, anti-Fas antibodies and growth factor withdrawal (Matsuyama S et al, 2000).

Bcl-2 over-expression inhibits CD95 receptor-induced acidification in Jurkat cells (Petit F et al, 2001). A very interesting observation has been reported recently, where intracellular alkalinization has been proposed to induce tumorigenesis by destabilization of a mutant p53 tetramer (DiGiammarino J et al, 2002). Although
association between alkaline intracellular pH and tumorigenic transformation is well documented in scientific literature, there are conflicting reports concerning whether tumorigenic transformation follows intracellular alkalinization or vice versa. Interestingly, there is increasing evidence suggesting that alkalinization is an early key event for the establishment and maintenance of oncogenic transformation (Reshkin SJ et al, 2000; Gillies RJ et al, 1990).

I.4. Intracellular pH regulation

Normal functioning of cell metabolism occurs within a restricted intracellular pH (pHi) range. In most cells pHi is maintained at a value of about 7.0. Variations in ambient pH alter the occupancy of acidic and basic groups on various cellular proteins and other molecules. Thus, for the normal functioning of the cell, pHi has to be very tightly regulated. There are various mechanisms operating in a cell to regulate pH within this very narrow physiological range. Few important examples are H⁺ buffering by intracellular buffers, sequestering of H⁺ into intracellular compartments, CO₂ diffusion across the cell membrane, carbonic anhydrase activity, and last but not the least transport of acid/base equivalents across cell membrane via specialized transporters.

In fact many cells face a constant acid load due to metabolic acid production and leakage of H⁺ ions from intracellular compartments. Intracellular buffers can blunt this acid load but they will not be able to restore pHi to its original value. The most effective way to deal with this is through membrane-bound transporters that operate with a slower time course. Most cells have one or more types of Na⁺-driven antiporters in their plasma membrane that help to maintain the pHi. These proteins use the energy stored in the Na⁺ gradient to pump out excess H⁺.
I.4.a. Plasma membrane pH\textsubscript{i} regulators

Several transport proteins within the cell membrane are specialized to actively transport acids and bases across the membrane. Two mechanisms are used: either H\textsuperscript{+} is directly transported out of the cell or HCO\textsubscript{3}\textsuperscript{-} is brought into the cell to neutralize H\textsuperscript{+} in the cytosol. There are three major classes of transporters in mammalian cells. Na\textsuperscript{+}-H\textsuperscript{+} exchanger (NHE) responds to cellular acidification by extruding H\textsuperscript{+} ions out of the cell in exchange for Na\textsuperscript{+}. Second class of membrane transporters includes HCO\textsubscript{3}\textsuperscript{-} dependent transporters (Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger, Na\textsuperscript{+} - HCO\textsubscript{3}\textsuperscript{-} cotransporter). Na\textsuperscript{+}-driven Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger couples an influx of Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} to an efflux of Cl\textsuperscript{-} and H\textsuperscript{+} (so that NaHCO\textsubscript{3} comes in and HCl goes out). A Na\textsuperscript{+}-independent Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger also has an important role in pH\textsubscript{i} regulation. Like the Na\textsuperscript{+}-dependent transporters, the Cl\textsuperscript{-}-HCO\textsubscript{3}\textsuperscript{-} exchanger is regulated by pH\textsubscript{i} but the movement of HCO\textsubscript{3}\textsuperscript{-}, in this case, is normally out of the cell, down its electrochemical gradient.

Third class of membrane-bound transporters comprises of H\textsuperscript{+}-ATPases or proton pumps. Except for H\textsuperscript{+}-ATPases, none of the carriers has a direct requirement for ATP (Molecular Biology of the Cell 4th ed. 2002).

In a given situation, pH\textsubscript{i} regulation would depend upon a balance between the rate of acid generation and its elimination or extrusion from cell. Steady state pH\textsubscript{i} is given by the balance between acid production, acid efflux and acid influx (Leem et al, 1999). When cells are faced with an acid load or challenge various mechanisms to maintain pH\textsubscript{i} come into effect.

Indeed, several studies have demonstrated that active extrusion of H\textsuperscript{+} by the Na\textsuperscript{+}-H\textsuperscript{+} exchanger (NHE) is one of the major regulatory mechanisms used by most if not all mammalian cell types. Most interestingly, NHE has been suggested as the main pH\textsubscript{i}
Fig. F. Various plasma membrane-bound pHi regulators.

(A) H⁺-lactate co-transporter, (B) H⁺ Channel, (C) Cl⁻-HCO₃⁻ exchanger, (D) Na⁺-H⁺ exchanger, (E) Na⁺ driven Cl⁻-HCO₃⁻ exchanger (F) Na⁺-HCO₃⁻ cotransporter.
regulator in transformed cells. NHE isoform 1 which is ubiquitously expressed in most mammalian cells has been extensively studied.

I.5. Na\(^+\)-H\(^+\) Exchanger (NHE)

The Na\(^+\)-H\(^+\) exchangers (NHEs) are a family of membrane glycoproteins which transport H\(^+\) out of the cell in exchange for Na\(^+\) with a stoichiometry of 1:1. In mammalian cells, the NHE family consists of nine isoforms, NHE-1 to NHE-9. Whereas most of the isoforms are restricted in their distribution and function, NHE-1, the first one of the isoforms to be cloned, is ubiquitously distributed (Sardet C et al, 1989; Brett CL et al, 2002; Goyal S et al, 2003; Numata M and Orlowski J, 2001; Putney LK et al, 2002). NHE1 is discussed in detail in the next sections. Unlike NHE1, other isoforms are restricted in their subcellular and tissue distribution.

The isoforms NHE2 to NHE5 are located in plasma membrane but have specific tissue distribution patterns. NHE2 and NHE3 are primarily located in apical membrane of epithelia, being most abundant in stomach and intestine (Collins JF et al, 1993; Wang Z et al, 1993). NHE3, in addition, is also found in kidney. NHE4 is expressed predominantly in stomach, kidney medulla, and hippocampus (Bookstein C et al, 1997). NHE5 is most abundant in brain but is also present in other non-epithelial tissues including spleen, skeletal muscle and testis (Orlowski J and Grinstein S, 1997).

The remaining four isoforms of NHE (NHE6 to NHE9) are distributed in intracellular compartments in humans. Mostly they are localized in Golgi and post-Golgi endocytic compartments. Recently it has been proposed that these isoforms contribute to the maintenance of the unique acidic pHs of the Golgi and post-Golgi compartments in the cell (Numata M and Orlowski J, 2001; Norihiro N et al, 2005).
**1.5.a. NHE1: Basic Structure**

NHE1 is the housekeeping protein which is present in most of the cells, if not all. It’s a membrane glycoprotein of approximately 100 kDa containing around 815 Amino Acids (membrane domain of about 500 amino acids and a large cytoplasmic “tail” of about 300 amino acids). Homology of NHE1 across various species is very high, but for the other isoforms (NHE2-NHE9) homology varies between 25-70%. NHE1 displays inhibition by diuretic amiloride and its derivatives. It has twelve transmembrane domains, with both N- and C- terminals in the cytosol. C-terminal forms a long tail which contains binding sites for many regulatory proteins and a putative phosphorylation site (Fig. F), however the exact structural details of NHE1 still remain unknown (Hunte C et al, 2005).

Several of the transmembrane (TM) domains of NHE1 are important in its function (Fleigel L, 2005). TM IV seems to be the most important segment for NHE1’s affinity for Na\(^+\) and its sensitivity to NHE1 inhibitors. TM VI and VII are important for activity, and TM XI is essential in targeting NHE1 to the cell membrane. NHE1 protein once synthesized in the cell undergoes posttranslational modifications, mainly glycosylation, before being targeted to the cell membrane. The large cytoplasmic “tail” of NHE1 has a putative phosphorylation site and binding sites for various regulatory proteins. Kinases known to phosphorylate NHE1 include Erk 1/2, p90\(^{rsk}\), p160 ROCK, p38 and Nck-interacting kinase (Khaled AR et al, 2001; Tominaga T and Barber DL, 1998). The Na\(^+\)/H\(^+\) exchanger is maximally active at low intracellular pH (pH <6.5). Its activity declines as the intracellular pH increases and binding of regulatory proteins has been shown to shift the pH dependence towards a more alkaline range.
Fig. G. Structure of mammalian NHE-1.

Topology of the NHE1 is shown after Wakabayashi et al. (2000). It’s a large protein having 12 trans-membrane domains. Tentative binding sites of some important regulatory proteins on long cytoplasmic “tail” are shown. The region of phosphorylation by regulatory kinases is also indicated.

IL, Intracellular Loop; EL, Extracellular Loop; CaM, Calmodulin; CHP, Calcineurin Homologous Protein; ERM, Ezrin Radixin and Moesin; TC, Tescalcin.
I.5.b. NHE1: Major Functions

I.5.b.i. pH and cell volume regulation

Na\(^+\)/H\(^+\) exchange activity is centrally important in many physiological processes, the most important role being regulation of intracellular pH. The Na\(^+\)/H\(^+\) exchanger is stimulated by a drop in intracellular pH. Activation of NHE leads to increased acid extrusion from the cell resulting in cytoplasmic alkalinization. The energy of Na\(^+\) gradient is used to catalyze the electro-neutral exchange of one Na\(^+\) for one H\(^+\). In doing so NHE also plays an important role in cell volume regulation after osmotic shrinkage (Shrode L et al, 1996). Upon exposure of cells to hyperosmotic solutions, Na\(^+\)/H\(^+\) exchanger shows increased activity that result in cytosolic alkalinization and increase in cell volume. Although NHE1 is not the only one to regulate cell volume, it plays an important role in cell volume regulation.

I.5.b.ii. Cell proliferation and differentiation

Na\(^+\)/H\(^+\) exchanger’s role in cell proliferation has been long known. In addition to protecting cells from intracellular acidification, NHE has also been shown to initiate shifts in pH that stimulate growth of cells and plays a role in malignant transformation (Reshkin SJ et al, 2000).

Although NHE1 has been long associated with cell proliferation but the exact mechanism remains unknown. In a recent study, NHE1 activity and pH have been shown to regulate the timing of G\(_2\)/M entry and transition, thus affecting the cell cycle (Putney LK and Barber DL, 2003).

Increased activity and increased levels of expression of NHE are also important in cell differentiation (Rao GN et al, 1993; Dyck JRB and Fliegel L, 1995), however there are conflicting reports regarding the causal relationship of increased promoter activity of NHE1 to differentiation (Vairo G and Hamilton JA, 1993).
Fig. H. Physiological Functions of NHE-1.

Figure adapted from Larry Fliegel (2005). (A) pH regulation, (B) Hormones like epidermal growth factor (EGF) and angiotensin II (Ang II) can activate NHE1. This leads to increased cell growth and cell differentiation. (C) Activation of NHE1 during ischemia and reperfusion results in increased [Na+] inside the cell that leads to increased intracellular [Ca++] through the Na+/Ca+ exchanger (NCE) and ultimately cell damage and cell death. This is the mechanism proposed for myocardial damage. (D) NHE1 binding to ERM proteins regulates cytoskeleton and plays an important role in cell migration.
I.5.b.iii. Cell motility

Interestingly, NHE1 has been found to play other key roles in the cell. It acts as a structural anchor that is involved in organization of the cytoskeleton (Denker SP et al, 1998), thus playing an integral role in cell shape and movement. Actin filaments play a pivotal role in determining the shape and motility of a cell and also associate with the dynamic extensions like lamellipodia (Lagana A et al, 2000). NHE1 interacts directly to actin-binding proteins ezrin radixin and moesin (ERM) (Denker SP et al, 2000). Regulation of cell volume and shape may be an important factor in determining the metastatic potential of a tumor.

I.5.b.iv. As a plasma membrane scaffold

In addition to its function as pH\textsubscript{i}/cell volume regulator, and cytoskeletal interaction, a third major function of NHE1 has been recently proposed. Like other integral membrane proteins, NHE1 may act as plasma membrane scaffold in the assembly of signaling complexes (Baumgartner M et al, 2004). Some very important proteins that interact with NHE1 include phosphotidylinosotol 4, 5-bisphosphate (PIP\textsubscript{2}); calmodulin (CaM); ezrin, radixin and moesin (ERM); heat shock protein 70 (Hsp70), Rho kinase 1 (ROCK1) and p90-ribosomal protein S6 kinase (p90-RSK). NHE1 promotes protein interactions, assembles signaling complexes in specialized membrane domains, and coordinates divergent signaling pathways.

I.5.b.v. Cell injury

NHE1 has been most extensively studied for its role in heart muscle. NHE1 gets activated when there is ischemic injury to the myocardium. This activity is more pronounced during the phase of re-perfusion. Increased Na\textsuperscript{+} that gets accumulated in the cell activates the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Na\textsuperscript{+} then leaks out of the cell in exchange for Ca\textsuperscript{2+} ions. Increased concentration of Ca\textsuperscript{2+} ions in the myocardium leads to cell
injury and death. Because of this function of NHE1, many NHE1 selective inhibitors have been designed and some have even entered into clinical trials. Major known functions of NHE1 are summarized in Fig. D.

I.5.c. NHE1: Regulation

Although fluxes through the NHE are driven by chemical gradients of \( \text{Na}^+ \) and \( \text{H}^+ \) and do not require energy, the increase in transport activity of NHE1 is often associated with phosphorylation of a number of serine residues within the distal C-terminal cytoplasmic domain. The major intracellular signal that leads to increase in NHE1 activity is an increase in \( \text{H}^+ \) concentration (Lacroix J et al, 2003). In addition, there are sites for binding to various proteins. Important regulatory proteins that have been found to bind to NHE1 C-terminal include calmodulin (Bertrand BS et al, 1994), HSP70 (Silva NL et al, 1995), tescalcin (Li et al, 2003), carbonic anhydrase II (CAII) (Putney LK et al, 2002), and CHP, a calcineurin homologue (Lin and Barber, 1996). Associated proteins or lipids are likely to affect NHE activity, for example calmodulin and CAII are stimulatory whereas tescalcin is inhibitory to NHE1 activity. Binding of regulatory proteins may also shift the pH dependence of NHE1 activity to a more alkaline range. One important fact to remember is that these regulatory proteins may themselves be subject to phosphorylation. Polyphosphoinositides are ubiquitous constituents of plasma membrane, where they have been shown to exert modulatory effect on the activity of many ion transporters. Phosphotidylinositol 4,5-bisphosphate (PIP\(_2\)) is an important member of this family. Recently, it was shown that PIP\(_2\) exerts a regulatory role on NHE1 even in ATP-depletion conditions (Aharonovitz O et al, 2000). Members of the ezrin/radixin/moeisin (ERM) family were recently reported to interact with NHE1 (Denker SP et al, 1998), and it is known that they are also capable
of binding to PIP<sub>2</sub>. Therefore PIP<sub>2</sub> may play an important role in regulating NHE activity either by its direct binding or through ERM proteins.

However, there is strong evidence that NHE1 activity is regulated by phosphorylation dependent as well as -independent mechanisms (Putney LK et al, 2002). This has been elegantly demonstrated in experiments where NHE1 phosphorylation was abolished by deletion of the C-terminal serine residues. When subjected to growth factor stimulation, cells expressing the C-terminal truncated NHE1 still retained up to 50% of the transport activity, and were completely insensitive to the change in activity following osmotic shrinkage (Putney LK et al, 2002). In addition, kinase-independent activation of the antiporter is further corroborated by findings that ATP depletion does not completely block acid stimulated NHE-1 activity (Cassel D et al, 1986). A more direct evidence to support dynamic regulation of NHE1 transcription is provided by several reports, e.g. (a) upon growth factor in vascular smooth muscle cells (Rao GN et al, 1992), (b) by acid in renal epithelial cells (Moe OW et al, 1991), (c) during respiratory and metabolic acidosis in rat kidney cells (Krapf R et al, 1991), (d) with phorbol ester-induced differentiation (Rao GN et al, 1991), and (e) during the process of cellular proliferation (Grinstein S et al, 1989; Rotin D et al., 1989).

**I.5.d. NHE1: Regulation of gene expression**

NHE1 gene is located on chromosome 1 in humans, spans approximately 70 kilobases. The coding region is divided into 12 exons and 11 introns. The promoter/enhancer region contains a TATA box, four GC boxes, two CAAT boxes, three AP-1 sites and a cyclic AMP response element (Miller RT et al, 1991). NHE1 gene shows high homology across various species; mouse NHE1 gene is more than 90% homologous to human gene.
Various studies have shown that NHE mRNA levels are increased in response to chronic acid loading and different experimental triggers that lead to cellular differentiation. Mitogenic stimulation of NHE1 leads to increased gene transcription and increased expression of NHE1 protein on cell surface (Besson P et al, 1998). Other evidences have suggested that mRNA levels of the exchanger are increased during cellular proliferation in intact tissues (Elsing C et al, 1994).

Transcription factor AP-2 or an AP-2-like protein has been shown to be involved in regulation NHE1 gene during differentiation of P19 cells (Dyck JRB and Fliegel L, 1995). The AP2 transcription factor family is a set of developmentally regulated, retinoic acid inducible genes composed of four related factors AP2α, AP2β, AP2γ, and AP2δ. AP2 factors orchestrate a variety of cell processes including apoptosis, cell growth, and tissue differentiation during embryogenesis. They have also been shown to regulate the expression of genes in various tissues and tumors. Several lines of investigation have led to the conclusion that AP2 is a tumor suppressor gene.

Recent cloning of NHE1 gene has allowed better insights into the regulation of NHE1 promoter. Scientists have observed that a step-wise reduction in the 5’ end of the NHE1 promoter leads to a gradual reduction of promoter activity; however, this effect varies amongst different cell types. In addition, DNA foot-printing experiments have suggested that many regions of the promoter bind proteins of nuclear extract (Kolyada AY et al, 1994; Yang W et al, 1996). A highly conserved poly (dA dT)-rich region also seems to play an important role in regulation of NHE1 expression (Yang W et al, 1996). Several putative proximally acting transcription factors are involved in regulation of basal NHE1 expression including AP-1, AP-2 and C/EBP (Dyck JRB and Fliegel L, 1995; Miller RT et al, 1991; Kolyada AY et al, 1994).
Fig. I. Basic structure of proximal part of NHE1 promoter (Highlighting the various truncated regions of promoter used in this study). There is >90% homology between mouse and human NHE1 gene.
In addition to AP-2, several other transcription factors have been proposed. One such group is the chicken ovalbumin upstream promoter-transcription factor (COUP-TF). COUP-TFs are orphan receptors involved in regulation of embryonic development and neuronal cell fate determination. COUP-TF I and II regulate NHE1 gene expression through a nuclear hormone responsive element in NIH3T3 and L6 cells (Fernandez-Rachubinski F et al, 2001).

Recent studies have also shown that the Na+/H+ exchanger is regulated in response to thyroid hormone. Reduction in circulating thyroid hormone levels reduces the amount of both protein and mRNA of NHE1. The NHE1 promoter contains a TR-α1 binding site. This regulation of the NHE1 promoter by thyroid hormone is proposed to be responsible for postnatal changes in expression of the Na+/H+ exchanger (Slepkov E and Fliegel L, 2004)

In summary, mitogenic signals that lead to cell growth also lead to NHE1 gene activation. Many transcription factors that are important during cell development and differentiation activate NHE1 gene. Evidence suggests that NHE1 activation at both transcription and protein level plays an important role in regulating its function.

I.5.e. NHE1: Pathological Functions

1.5.e.i. Role in Myocardium

Much work on NHE1 has been done in context to its possible role in mammalian heart. Evidence suggests the pH regulation activity of NHE1 plays a significant role in mediating the damage that occurs to the human myocardium during ischemia and reperfusion. Increased NHE1 activity during ischemia results in accumulation of intracellular Na⁺ which in turn results in reduction of Ca²⁺ extrusion by the Na⁺/Ca²⁺
exchanger (Karmazyn M et al, 1999). Excess Ca\(^{2+}\) therefore accumulates in the cell and causes cell necrosis, contracture and cardiac arrhythmias.

Amiloride (a K\(^{+}\)-sparing diuretic), in addition to its ability to block a conductive Na\(^{+}\) channel and the Na\(^{+/Ca^{++}}\) exchanger, was the first drug described as NHE inhibitor. In a bid to develop more selective NHE1 inhibitors many amiloride derivatives have been designed. Later, several acylguanidines were prepared with even greater potency and NHE selectivity, including HOE-694, cariporide and eniporide (Masereel B et al, 2003). Amiloride derivatives and other NHE inhibitors were found successful in blocking the damage to myocardium in animal models.

However, a recent clinical trial, the GUARDIAN trial, showed that cariporide (a selective NHE1 inhibitor) provides cardioprotective effect only in one subgroup of patients, those with coronary artery bypass surgery (Avkiran M and Marber MS, 2002). Further validation of NHE1 inhibitors as cardioprotective agents following an ischemic event in the heart is ongoing.

**1.5.e.ii. NHE1: Role in Tumorigenesis**

Several studies have shown both increased *message* and *protein* of NHE1 in transformed cells. Apart from its role as a principal regulator of pH\(_{i}\) and cell volume, NHE1 has been implicated in cell proliferation and transformation, thereby linking alkaline intracellular milieu with processes of proliferation and transformation (Putney LK et al, 2002). Rapid stimulation of the NHE1, with consequent alkaline pH\(_{i}\), appears to be a universal response of quiescent cells to growth-promoting factors and mitogens (Schuldiner S and Rosengurt E, 1982; Cassel D et al., 1983; Moolenaar WH et al, 1983; Mills GB et al, 1985). Moreover, tumor cells deficient in Na\(^{+}/H^{+}\) exchange activity either fail to grow or show severely retarded growth when implanted in immuno-deficient mice (Rotin D et al, 1989). It also might play a role in
moderating apoptosis, at least in human leukemic cells (Rich IN et al. 2000). With respect to Fas receptor-induced apoptosis, a recent study has demonstrated that the related cytoplasmic acidification is partly due to inhibition of NHE1 (Lang F et al, 2000). The phorbol ester 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA) has been long known to induce intracellular alkalinization via NHE1 activation in various cells. It has recently been reported to rescue T lymphocytes from Fas-mediated apoptosis via activation of p90rsk, resulting in phosphorylation and inactivation of BAD, a proapoptotic member of Bcl-2 family.

In an in vivo study, when Ras-transformed fibroblasts were implanted in nude mice very interesting observations were made (Pouyssegur J et al, 2001). Ras-transformed pgi- cells (not forming lactic acid) formed tumors like wild type transformed cells (100% incidence). The disruption of NHE1 however, strongly reduced tumor incidence to about 20%. This study lead to the conclusion that over-production of lactic acid is detrimental for tumor development and that NHE1, by controlling pH\textsubscript{i}, plays a key role in cell survival/proliferation and tumor growth.

Paclitaxel, a well known chemotherapeutic drug, was recently shown to induce apoptosis in breast cancer cells in an NHE1-dependent fashion (Reshkin SJ et al, 2003). In this model, paclitaxel treatment resulted in activation of protein kinase A and p38 MAPK, and inhibited the activity of NHE1. Paclitaxel belongs to the “texanes” group of anti-cancer drugs which are active against cancers that are refractory to other types of chemotherapy. Major mode of action of taxanes is thought to be their ability to bind to tubulin leading to G\textsubscript{2}/M arrest of the cell and subsequently apoptosis. Down-regulation of NHE1 activity by using a specific inhibitor (DMA) synergistically potentiated the paclitaxel-induced apoptosis at very low concentrations.
Recently, it has been shown by Denker and Barber that NHE1 acts as a structural anchor and is involved in organization of cytoskeleton. This structural interaction of NHE1 with cytoskeleton is independent of its ion exchange function. This anchoring occurs via direct structural interaction of NHE1 with actin-binding proteins ezrin, radixin and moesin (Denker SP et al, 2000).

Recruitment of ERM proteins by NHE1 regulates Akt-dependent cell survival (Karen LW et al, 2004). Cell migration is a multi-step process that requires spatial asymmetry which is stimulated by Rho GTPases, phosphoinositides and actin polymerization. Disrupting NHE1 function leads to impaired polarity of cells and their inability to migrate in response to these stimuli. However, this role in cell migration seems to require both ion translocation and cytoskeletal anchoring function of NHE1 (Denker SP and Barber DL, 2002b). The central role NHE1 plays in cell movement and migration has been proposed as a determining factor of the metastatic potential of a tumor (Cardone RA et al, 2005).

pHi and cell volume regulation are the principle functions of NHE1. In addition, it has been shown to play a role in cell growth, motility, differentiation and transformation. However, in the light of recent advances in our understanding of NHE1 functions, it may not be wrong to speculate that the role NHE1 plays in tumor cells may or may not be a dependent on its pH regulatory function.

I.6. Rac subfamily and NHE1

Numerous studies support a role for the small GTPases of the Ras family in proliferation, transformation and NHE1 activation (Hooley R et al, 1996; Voyno-Yasenetskaya T et al, 1994). Although, the signaling pathways acting downstream of these GTPases to stimulate NHE1 are distinctly different, they all lead to the
activation of kinases. For example, Gaq acts through a protein kinase C-dependent mechanism, Ras acts through a Raf-1 and mitogen-activated protein kinase kinase (MAPK)-dependent mechanism and Ga13 employs a MEK kinase (MEKK1)-dependent mechanism for NHE-1 stimulation. In addition, GTPases of the Rho family, Rac, Cdc42 and Rho also mediate Ga13 activation of NHE1 through phosphorylation of the exchanger (Denker SP et al, 2000a and 2000b; Hooley R et al, 1996; Lin X and Barber DL, 1996; Tominaga T and Barber DL, 1998).

However, considering the fact that induction of proliferation and transformation by Rho GTPases occurs over a longer time-scale compared to the short-term changes of the actin cytoskeleton and the implication that these events require transcriptional activation, it is plausible that the small GTPases such as Rho, Rac or Cdc42 directly or indirectly influence gene transcription.

I.7. Conclusion

In summary, NHE1 is a ubiquitously expressed protein in most cells and it performs at least three important functions in a cell: 1) Intracellular pH/Cell volume regulation; 2) acts as a cytoskeletal anchor by its direct interaction with ERM proteins; and 3) as a membrane scaffold in the assembly of signaling complexes. Tumor cells display increased expression and activity of NHE1. NHE1 has been implicated in cell proliferation, survival, and transformation, although the exact mechanism remains largely unknown (Putney LK et al, 2002). Growth factors such as serum, EGF and phorbol ester myristyl acetate (PMA) have all been shown to activate NHE-1 promoter activity (Besson P et al, 1998). In addition, another characteristic of tumor cells is a higher metabolic rate and an altered redox state. A slight pro-oxidant intracellular environment also shares the pro-survival and proliferative properties of
NHE1. Involvement of ROS in NHE1 activation merits to be investigated as phorbol esters have been shown to activate NHE1 by $\text{O}_2^-$ (Shibanuma M et al, 1988). AP-2 transcription factor that has been shown to regulate NHE1 gene expression belongs to the family of redox-regulated transcription factors. Intracellular alkalinization seems to be an upstream event in the apoptotic cascade, and upon initiation of apoptosis, is followed by intracellular acidification. The model that we propose is of a long-term maintenance of an alkaline pH (for example by adjusting the pH set-point of NHE1 to a higher pH) in a tumor cell.

So, our question simply was whether their exists any functional relationship between increased $\text{O}_2^-$ production in the cell and regulation of NHE1 expression. And if so, does this interaction enable tumor cells to evade cell death triggers.
Chapter II

Materials and Methods

II.1. Chemicals

Lucigenin, staurosporine, diphenylene iodonium (DPI), Diethylthiocarbamate (DDC), 5-(N-Ethyl-isopropyl) amiloride (EIPA), Nigericin and crystal violet were obtained from Sigma Chemical Co (St Louis, MO), etoposide from Clonetech Laboratories, Inc. (Palo Alto, CA). Etoposide, staurosporine and DPI were diluted in dimethylsulfoxide (DMSO). Stock solutions were at least 1000x. 1/1000 dilutions of DMSO did not alter cell viability or interfere with apoptotic cell death. Hydrogen peroxide ($H_2O_2$) was from Merck. Cm- BCECF was from molecular probes.

II.2. Cells

All cell lines used in this study were adherent cells, and for most of the assays cells were used at around 70% confluency. NIH3T3 mouse fibroblast cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine and 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA).

NIH3T3 cells stably transfected with a 1.1-kb proximal fragment of the mouse NHE1 promoter/enhancer inserted 5′ to a luciferase gene (NIH3T3 1A8 and NIH3T3 Luc) (Besson et al., 1998) or a Chloramphenicol Acetyl Transferase (CAT) gene (NIH3T3 CatC2 and NIH3T3 CatB3) (Besson et al., 1998) were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine, 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA) and 0.25mg/ml G418 (Roche Diagnostics Corporation, In, USA).
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Type</th>
<th>Culture Medium</th>
<th>Selective Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH3T3</td>
<td>Mouse</td>
<td>Normal Fibroblast</td>
<td>RPMI</td>
<td>-</td>
</tr>
<tr>
<td>NIH3T3 1A8</td>
<td>Mouse</td>
<td>Normal Fibroblast</td>
<td>MEM</td>
<td>G418</td>
</tr>
<tr>
<td>NIH3T3 Luc</td>
<td>Mouse</td>
<td>Normal Fibroblast</td>
<td>MEM</td>
<td>G418</td>
</tr>
<tr>
<td>NIH3T3 B3</td>
<td>Mouse</td>
<td>Normal Fibroblast</td>
<td>MEM</td>
<td>G418</td>
</tr>
<tr>
<td>NIH3T3 C2</td>
<td>Mouse</td>
<td>Normal Fibroblast</td>
<td>MEM</td>
<td>G418</td>
</tr>
<tr>
<td>MCT</td>
<td>Mouse</td>
<td>Normal Renal Tubular</td>
<td>RPMI</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epithelial Cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L6</td>
<td>Rat</td>
<td>Normal Myoblast</td>
<td>DMEM</td>
<td>-</td>
</tr>
<tr>
<td>M14 plRES</td>
<td>Human</td>
<td>Malignant Melanoma</td>
<td>DMEM</td>
<td>Hygromycin</td>
</tr>
<tr>
<td>M14 RacV12</td>
<td>Human</td>
<td>Malignant Melanoma</td>
<td>DMEM</td>
<td>Hygromycin</td>
</tr>
<tr>
<td>U87</td>
<td>Human</td>
<td>Malignant Glioblastoma</td>
<td>RPMI</td>
<td>-</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Human</td>
<td>Malignant Prostate Cancer</td>
<td>RPMI</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Various Cell Lines used in the project.
Mouse kidney tubular epithelial cells stably transfected with a 1.1-kb proximal fragment of the mouse NHE1 promoter/enhancer inserted 5’ to a luciferase gene (MCT 1.1kb) were maintained in RPMI medium supplemented with 5% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine, 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA) and 0.25mg/ml G418 (Roche Diagnostics Corporation, In, USA).

Rat muscle cell lines stably transfected with a varying length of proximal fragment of the mouse NHE1 promoter/enhancer inserted 5’ to a luciferase gene (L6 1.1Kb, L6 0.9Kb, L6 0.5Kb, L6 0.2Kb, L6 0.18Kb and L6 AP2+) (Yang et al., 1996) were maintained in DME medium supplemented with 5% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine, 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA) and 0.25mg/ml G418 (Roche Diagnostics Corporation, In, USA).

M14 human melanoma cells stably transfected with a constitutive activated form of Rac1 (M14RacV12) or the vector control (M14pIRES) (Pervaiz et al., 2001) were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine, 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA) and Hygromycin (Roche).

T24 human bladder carcinoma cell line was maintained in McCoy’s Modified Medium supplemented with 5% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine and 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA).

U87 human malignant glioma cell line was maintained in RPMI medium supplemented with 10% fetal bovine serum (Hyclone, Irvine, CA), 1% glutamine and 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA).
II.3. cDNA Plasmids and transfections

The partial myc-tagged loss-of-function mutants of Rac1 containing specific amino acid substitutions in the activated RacV12 background used in this study were all cloned in the pIREShyg vector as described previously (Pervaiz et al., 2001). The pECE-NHE1 plasmid was obtained from Pouyssegur. All transfections were performed using CalPhos™ mammalian transfection kit (Clontech Laboratories, Inc., Palo Alto, CA). In brief, 0.3x10^6 cells/well were plated in 6-well plates 24 hours prior to transfection. Two hours before transfection, the cell culture medium was changed with DMEM supplemented with 5% FBS. To prepare the transfection mixture, 6 µg of DNA, (5 µg of the required plasmid and 1 µg of the pCMV β-gal plasmid) was diluted in sterile ddH₂O in a microfuge tube, followed by the addition of 12.4 µl of 2.5 M CaCl₂ in a total amount of 100 µl/well. Two solutions were prepared as following:

<table>
<thead>
<tr>
<th>Solution A</th>
<th>DNA (6 µg)</th>
<th>CaCl₂</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>? µl</td>
<td>12.4 µl</td>
<td>To make up 100 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution B</th>
<th>2 x HBS (HEPES Buffered Saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Solution A was then mixed drop-wise into solution B while gently vortexing. The transfection mixture was incubated at room temperature for 20 minutes.

The mixture (200 µl) was added to the medium of each well drop-wise. Plates were shaken gently and put back in the incubator. This step was important because DNA precipitates are heavy in weight they may settle in the center or periphery of the well.
rather than being distributed evenly. After 16 hours of incubation, cells were washed twice with 1 x PBS. Glycerol Shock was performed to enhance the transfection efficiency. Two ml of 15% glycerol in PBS was added per well for 90 seconds. Cells were quickly washed with PBS twice and normal medium was replaced. After 24-48 hours of incubation, cells were harvested for appropriate assays.

β-gal activity was measured using the Galacto-Star mammalian reporter Kit (TROPIX, Bedford, MA).

II.4. β-Galactosidase Survival Assay

Co-transfection of cells was done with pCMV-β plasmid encoding for the β-galactosidase protein (β-gal). 16 hours later Glycerol Shock was done as described under transfection procedure. After 24 hours the apoptotic trigger was added for an additional 24 hours. Survival was calculated as: 

\[
\left( \frac{\beta\text{-gal activity/µg of protein of transfected cells incubated with the apoptotic trigger}}{\beta\text{-gal activity/µg of protein of transfected cells left for the same period without the apoptotic trigger}} \right) \times 100
\]

β-gal activity was measured using the Galacto-Star mammalian reporter kit.

II.4a. Measurement of β-Galactosidase activity

Lysate preparation:

Culture medium was removed and cells were washed twice with 1X PBS. 200 ul of Cell Lysis Buffer was added per well. Cells were incubated in the lysis buffer at room temperature for 15 minutes. Cells were detached from the plate using a cell scraper and the lysate was transferred to 1.5 ml microfuge tube.

Lysates were immediately used or stored at –80 °C for later use.
β-gal Assay:

Reaction Buffer was prepared by diluting Galacton-Star substrate 1:50 with Reaction Buffer Diluent. 10 µl of cell lysate was transferred to a luminometer tube and 300 µl of Reaction Buffer was added. The reaction mix was then incubated for 30 minutes at 30 ºC. Light emission signal was then measured for 10 seconds using the Turner TD 20/20 tube luminometer.

II.5. siRNA transfection

Transient siRNA transfections of cells were done using Oligofectamine (Promega). 0.15x10^6 cells/well were plated in 6-well plates (well diameter= 35 mm) 24 hours prior to transfection. (Cell density should be 40-50% at the time of transfection). Before starting the experiment, growth medium was removed and cells were washed once with PBS. 800µl of opti-MEM1 (without serum) was added per well and plates were put back in the incubator.

To prepare the transfection mixture, two solutions were prepared first. For Solution A, 200 µl of opti-MEM1 medium was added to a sterile microfuge tube and 6ul of siRNA was added to it and gently mixed. For Solution B, 6 µl of oligofectamine was added to 24 µl of opti-MEM1 in a separate microfuge tube. Solution A and B were mixed together gently by inverting and incubated at room temperature for 20 minutes. At the end of incubation period, 200 µl of transfection mixture was added drop-wise onto cells. 4 hours later, 500 µl of opti-MEM1 containing 3x the normal concentration of serum (which in case of NIH3T3 is 10% and M14 is 5%) was added per well.

24 hours later medium containing transfection complexes was replaced with fresh medium. Cells were assayed for siRNA efficiency 72-96 hours post-transfection. Generally, siRNA transfection efficiency was around 80-90%.
Following sequence was used to silence NHE-1:

5′- GAUAGGUUCCAUUGUGAUC

For negative control transfection an inverted sequence of luciferase gene was used to design our Control siRNA:

5′- AGCUUCAUAAGGCAGUAGCTT

II.6. Luciferase reporter gene assay

NHE-1 promoter activity in NIH3T3 1A8, MCT 1.1kb, L61.1kb was assessed using the luciferase reporter assay system according to the manufacturer’s instructions (Promega, Madison, WI). In brief, cell lysates were prepared using 200 µl/well of lysis buffer provided by the manufacturer. 20 µl of cell lysate was mixed with 100 µl of Luciferase substrate. Luminescence was measured for 10 seconds in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

II.7. CAT (Chloramphenicol acetyltransferase) ELISA

NHE-1 promoter activity was also assessed using an enzyme immunoassay to determine CAT expression. The Elisa assay was performed according to the manufacturer’s instructions (Roche Diagnostics Corporation, IN). 0.3 x 10^6 cells were plated in 35 mm (6-well) plates. The next day cells were either transiently transfected or trigger added depending on the experimental conditions. On the day of CAT assay, medium was removed and cells were washed thrice with pre-cooled PBS. Colorimetric enzyme immunoassay for quantitative determination of CAT was done using CAT ELISA kit from Roche Inc. All steps were done as described by the vendor. Absorbance was measured at 405 nm (reference wavelength:
490 nm) using a microplate ELISA reader. Standard curve was drawn and CAT enzyme was calculated in samples as pg/ml.

II.8. Measurement of steady state pHi

pHi was measured by loading cells with the fluorescent pH indicator BCECF-AM (2’,7’-bis(2-carboxyethyl)-5,6-carboxyfluorescein; Molecular Probes, Eugene, OR). Cells were plated in 6-well plates, after 24 hours they were subjected to different treatments and/or transfections and after appropriate time pHi measurement was undertaken. Cells remained attached to the 6-well plates throughout the pHi measurement assay. Briefly, cells were washed once with HBSS (Sigma, St. Louis, MO) before being incubated in 1 ml of HBSS containing 10ml of 1mM BCECF-AM at 37°C. Following 30 minutes incubation in the dark, cells were washed once with HBSS and fluorescence immediately measured in a Spectrafluor Plus (TECAN Austria, GmbH, Grödig, Austria) with the excitation wavelength at 485nm and 430 nm, and emission at 535 nm. The ratio of BCECF fluorescence was then used to obtain pHi from a pH calibration curve. A pH calibration curve was generated for each cells treatment and pHi measurements were calculated from this reference curve. In order to generate a pH calibration curve, cells were loaded with BCECF-AM as above, washed once with HBSS and then resuspended in high K⁺ buffer (135mM KH₂PO₄, 20mM NaCl, and 110mM K₂HPO₄, and 20mM NaCl with a range of pH between 6.0 and 8.0). Immediately before measurement of BCECF fluorescence, cells were loaded with 10mM nigericin (1mM stock in absolute alcohol, Sigma, St Louis, MO), and fluorescence ratio measurements (535nm/595nm) of cells in nigericin-containing buffers of a range of pH were then used to relate fluorescence ratio measurement to pHi.
II.9. Measurement of acid load and pH\textsubscript{i} recovery (NHE-1 activity)

Initial steps were similar to the protocol described in previous section. Cells were washed and incubated with 5 $\mu$M of BCECF-AM in HCO$_3^-$-free HEPES buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES, pH 7.4) for 30 minutes at 37$^\circ$C. The cells were washed and fluorescence of BCECF was measured (excitation at 485 nm and 430 nm; emission at 535 nm). pH\textsubscript{i} was derived by determining the ratio of dual excitation measurement (485 nm: 430 nm) from a pH\textsubscript{i} standard curve. To calibrate the relationship between the excitation ratio (485 nm: 430 nm) and pH, cells loaded with 5 $\mu$M BCECF-AM were incubated with 10 $\mu$M nigericin for 3 minutes in high K\textsuperscript{+} buffer of pH 6.4-8.0, obtained by mixing pH 4 buffer (140 mM KH$_2$PO$_4$ and 20 mM NaCl) and pH 9 buffer (70 mM K$_2$HPO$_4$ and 20 mM NaCl) and fluorescence measurement was taken. After measurement of pH\textsubscript{i}, the cells were acid loaded with HCO$_3^-$-free HEPES buffer containing 30 mM NH$_4$Cl, pH 7.4 for 8 minutes (the osmolarity of this buffer has been adjusted). The cells were then exposed to Na\textsuperscript{+}-free, HCO$_3^-$-free HEPES buffer (135 mM N-methyl glucamine, 5 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 5.5 mM glucose, 10 mM HEPES, pH 7.4) for 30 seconds and subsequently washed with HEPES buffer. The pH recovery from acid load was monitored for 15 minutes and NHE-1 activity was determined by the rate of pH\textsubscript{i} recovery.

II.10. Western blot analysis of NHE-1 protein

Cell lysates were prepared using 300 $\mu$l/well of RIPA/NP-40 lysis buffer (5 mM Tris pH7.4, 30 mM NaCl, 1 mM PMSF, 1 $\mu$g/ml aprotinin, and 0.5% NP-40). Lysate was denatured at 37$^\circ$C for 5 minutes, rather than the usual 95$^\circ$C. 300 $\mu$g of total protein
per sample was then subjected to 15% PAGE, transferred to nitrocellulose membrane and blocked 1 hour with 5% fat-free milk in Tris-buffered saline/0.05% Tween 20 (TBST). After three washes with TBST, the membranes were exposed to a 1:2000 dilution of a mouse anti-porcine NHE1 antibody (Chemicon International Inc., Temecula, CA, USA) at 4°C overnight. The anti-NHE-1 antibody used here cross-reacts with all vertebrate NHE-1 protein including human. Following three washes with TBST, the membrane was exposed to a 1: 10 000 dilution of goat anti-mouse IgG-HRP conjugate (PIERCE, Rockford, IL, USA) for 1 hour and washed repeatedly with TBST. Chemiluminescence was detected using the SuperSignal Substrate (PIERCE, Rockford, IL, USA).

II.11. Measurement of intracellular superoxide

A lucigenin-based chemiluminescence assay was used for the detection of intracellular \( \text{O}_2^- \) as described previously (Pervaiz and Clement, 2002). Briefly, cells were washed once with PBS, trypsinized, and centrifuged at 2000×g at room temperature for 1 minute. Pellets were resuspended and cells permeabilized in 400µl of 1X somatic cell ATP releasing reagent (Sigma-Aldrich, St. Louis, MO). 100 µl of 850µM lucigenin solution was injected automatically before the reading. Chemiluminescence was monitored for 180 seconds in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

II.12. Crystal Violet Assay

Culture medium was removed from 6-well plates by flicking. Cells were washed once with 1 ml of 1xPBS per well. 500 µl of Crystal Violet solution was added per well and incubated for 10 minutes at room temperature. Crystal Violet solution was then
removed by flicking the plate and wells were washed with tap water several times to get rid of the extra crystal violet solution. Plates were left to dry overnight. 1 ml of 1xPBS/1%SDS solution was added per well and left for 4 hours. Absorbance was then measured at 595 nm using a plate reader.

II.13. Caspase assay
Cells were lysed in 50 µl of chilled cell lysis buffer (10 mM Herpes, pH7, 4.2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 20 µg/ml leupeptin) and incubated on ice for 10 minutes. Then, 40 µl of 2x reaction buffer containing 10 mM DTT and 4 µl of 1 mM synthetic oligopeptide caspase 3, 7, 10 substrate DEVD-(7-amino-4-trifluoromethyl coumarin) (DEVD-AFC) were added to each sample and incubated at 37°C for 1 hour. Fluorescence was measured at excitation wavelength of 530 nm and emission wavelength of 485 nm inside spectrofluorometer. Protein concentration was determined using the coomasie plus protein assay reagent from Pierce (Pierce Chemical Company, Rockford, IL, USA).

II.14. Statistical Analysis
Statistical analysis was performed using the paired Student’s t-test with P < 0.05 considered significant.
Chapter III

Results

III.1.a Regulation of NHE-1 gene expression regulates cells' response to death triggers in NIH3T3 cells

The predominant function of NHE-1 is the regulation of intracellular pH (pHi). However, recent evidence strongly suggest that, in addition to regulating pHi, NHE-1 could be a critical regulator of cells’ sensitivity to death signals (Reshkin SJ et al, 2003; Wu KL et al, 2003). In order to test this observation in our system, NIH3T3 cells were transfected with increasing concentrations of pECE-NHE1 vector. In order to assess transfection efficiency as well as to calculate cell survival, cells were co-transfected with the pCMV-βGal plasmid which encodes for β-Galactosidase protein. β-Gal assay was used to assess cell death as it allows for the measurement of cell death in transfected cells only (Pervaiz S et al, 2001). Cells were co-transfected with the pCMV-βgal plasmid and either the pIRESempty vector (pIRES) as control or pECE-NHE1 (different concentrations). Using expression of β-gal as a marker, only transfected cells are analysed for response to apoptotic stimuli as non-transfected cells are excluded in assessment. Forty-eight hours post-transfection, sensitivity to apoptosis of pIRES or pECE-NHE1 transfected cells was assessed by measuring the β-gal activity in cells incubated with or without 0.5 µM staurosporine for an additional 18 h. Per cent survival was calculated as the (β-gal activity of transfected cells incubated with the drug/ β-gal activity of transfected cell incubated without the drug) x 100.

Fig 1A shows western blot analysis of NIH3T3 cells 48 hours post-transfection. With increasing amount of NHE1 cDNA up to 40% increase in NHE1 protein expression
Figure 1. Increased NHE-1 expression leads to inhibition of staurosporine-induced cell death in NIH3T3 cells.
NIH3T3 cells were co-transfected with increasing concentrations of the pECE vector encoding for the human NHE-1 cDNA (empty vector pIRES cDNA was added corresponding to varying pECE-NHE1 cDNA in order to keep the total cDNA amount constant) and 0.5μg of pCMVβ plasmid encoding for β-galactosidase as described in Materials and Methods. (A) Forty-eight hours post transfection level of NHE-1 expression was assessed by western blot analysis. Gel represents one transfection out of three. (B) Band intensity of NHE-1/β-actin ± SE from three different transfections has been averaged as % of pIRES transfected cells. (C) Cell death of NHE-1-transfected cells upon incubation with 0.5 μM staurosporin for 18 hours was assessed using the β-gal assay as described in Materials and Methods. Cell Death was calculated as [(β-gal activity/μg of protein of transfected cells incubated with staurosporine)/(β-gal activity/μg of protein of transfected cells incubated without staurosporine)]x 100. Results are shown as mean of 3 experiments ± SE. * P < 0.02
was observed (Fig 1B). When these cells were incubated with staurosporine for 18 hours and cell death was assessed by β-Gal assay, it can be seen in Fig 1C that dose-dependent increase in NHE1 protein expression correlated with the inhibition of staurosporine-induced cell death.

On the other hand, silencing of NHE1 gene was obtained by transfecting NIH3T3 cells with a specific NHE1 siRNA. Using siRNA to silence the target gene is being increasingly used as a molecular tool. It has proven to be a simple yet powerful tool to study functional genomics. In this technique, a short length RNA sequence (usually 21-23 amino acids) is designed to match the sequence of target gene (Elbashir SM et al, 2002). The siRNA binds to the target mRNA sequence and leads to its degradation thus leading to a functional knockdown of the gene of interest.

Following sequence was used to silence NHE-1:

5′- GAUAGGUUUUCCAUGUGAUC

A Control siRNA (Co siRNA) sequence was used as a negative control for the experiment. The sequence for Control siRNA must be designed as such that it does not recognize any mammalian mRNA. We used an inverted sequence of luciferase gene to design our Control siRNA (Co siRNA):

5′- AGCUUCAUAAGGCAGCAUGCTT

Fig 2A shows that upon transfection of NIH3T3 cells with the NHE1 siRNA, there was a significant drop in NHE1 expression level. Interestingly and as expected, decreased NHE1 expression led to increased sensitivity of the cells to staurosporine and etoposide-induced cell death (Fig 2B). Staurosporine and etoposide are well-documented triggers of apoptotic cell death. The doses of these drugs that were used in these experiments have been documented to induce apoptotic cell death (Pervaiz S
Figure 2. Silencing of NHE-1 gene leads to increased susceptibility to cell death in NIH3T3 cells.
Silencing the NHE-1 gene further supports a role for NHE-1 expression in cell response to apoptotic triggers. NIH3T3 cells were transfected with either Control siRNA (Co siRNA) or NHE1 siRNA (NHE1 siRNA) and pCMV-βgal cDNA. Forty-eight hours post-silencing (A) western blot analysis was done and (B) cell death was triggered for 18 hours with either 0.5 μM staurosporine (Sts) or 40 μM etoposide (Eto) before being assessed using the β-gal assay. Cell death was calculated as [(β-gal activity/μg of protein of transfected cells incubated with staurosporine or etoposide)/ (β-gal activity/μg of protein of transfected cells incubated without drugs)]x100 Results are shown as mean of 3 experiments ± SE. * P < 0.02

Figure 3. Manipulation of NHE-1 gene expression affects cells’ sensitivity to death triggers in NIH3T3 cells.
This figure shows a consolidation of data from Fig 1C and 2B. Staurosporine was used as the apoptotic trigger in these experiments.
et al, 2001). This was to avoid sudden and necrotic type of cell death and mimic physiological conditions more closely.

Initially cell death was assessed at various time points starting from 6 hours up till 24 hours. Eighteen hours time point was chosen for further experiments as it showed significant difference in cell death response.

One important point to note here is that β-Gal survival assay is particularly useful in NHE-1 over-expressing experiment because the transfection efficiency obtained in cDNA transfection experiments is generally agreed upon to be around 30%. On the contrary siRNA transfection generally yields a transfection efficiency of around 90%. β-Gal assay was used to assess cell death in both cDNA and siRNA transfection experiments. This was done purposely so that results obtained from both experiments could be compared to each other and could give a better index in terms of % cell survival. Thus, looking at the results from NHE1 over expression and silencing it can be deduced that cell death in response to staurosporine can vary from as low as 15% to 48%, depending on the level of NHE-1 protein expression (Fig 3).

Although no specific assays were performed to assess cellular proliferation, but simple observation of cells showed that silencing with NHE-1 siRNA did not result in any significant decrease in cell proliferation. This could be due to the fact that silencing NHE-1 with siRNA does not completely abolish NHE-1 protein (as opposed to NHE-1 knock out), and the effect of NHE-1 on proliferation could be independent of its cell survival function.

III.1.b Regulation of NHE-1 gene expression regulates cells’ response to death triggers in Tumor cells
Studies have demonstrated increased mRNA and protein levels of NHE-1 in transformed cells as compared to their normal counterparts. Reshkin et al. have recently demonstrated that NHE-1 protein expression is implicated in malignant transformation and the development of the transformed phenotype (Reshkin SJ et al, 2000). Thus, we decided to look at NHE-1 expression profile in tumor cells and see whether manipulation of its expression will have an effect on death sensitivity of these cells. Two tumor cell lines were used, U87 and LNCaP. U87 is a cell line derived from human glioblastoma which is one of the most aggressive and difficult to treat cancers. LNCaP is also a human cell line that has been derived from prostatic cancer. NHE1 silencing was obtained in both the cell lines by transfecting them with NHE1 siRNA (Fig 4A and Fig 6A). Using etoposide and staurosporine as apoptotic triggers and β-Gal assay to assess cell survival, it was noted that decreased NHE1 protein expression led to a significant increase in cell death in both U87 and LNCaP cells (Fig 4B and Fig 6B). To confirm apoptotic cell death, Caspase 3 assay was performed in U87 cells in order to delineate the type of cell death observed in these experiments. Caspase 3 is a downstream executioner caspase and has been classically used as a hallmark of apoptosis. Caspase 3 activity following incubation with etoposide was measured at different time points and it was observed that in NHE1 silenced cells caspase 3 activity was higher at 4 and 8 hours as compared to control cells, but after prolonged incubation (i.e, 18 to 24 hours) this difference could not be seen (Fig 5). Same observation was made in LNCaP cells (Fig 7) except that staurosporine and etoposide both triggers were used. Although the difference in caspase 3 activity between Control siRNA and NHE1 siRNA transfected cells is seen transiently, however the fact that NHE-1 silenced cells can activate caspase 3 earlier and in more robust fashion could represent more apoptosis-prone state of these cells.
Figure 4. Silencing of NHE-1 gene leads to increased susceptibility to cell death in U87 cells.
U87 cells were transfected with either Control siRNA (Co) or NHE1 siRNA (NHE1). Twenty-four hours post-silencing (A) western blot analysis was done and (B) cell death was triggered for 18 hours with either staurosporine (Sts) or etoposide (Eto) before being assessed using the β-gal assay as described in Materials and Methods. Results are shown as mean of 3 experiments ± SE. *P < 0.02

Figure 5. Time-dependent Caspase 3 (DEVDase) activity in NHE-1 silenced U87 cells treated with etoposide.
U87 cells were transfected with either Control siRNA (Co) or NHE1 siRNA (NHE1). Twenty-four hours post-silencing cell death was triggered with 10 or 20 μM of etoposide (Eto 10 and Eto 20). Lysates were prepared at different time points and DEVDase activity was measured. Results are shown as mean of 3 experiments ± SE. *P < 0.02 **P < 0.05
Another explanation for marginal differences in caspase 3 activities could be due to low level of caspase activation in these experiments.

Nevertheless, these results show that regulation of NHE-1 expression could be an important determinant of these cells’ sensitivity to apoptotic death. In summary, these experimental data confirm the role of NHE1 in resistance to cell death, and consequently when NHE1 gene is silenced leading to decreased NHE1 protein expression cells become more sensitized to apoptotic cell death.

**III.2.a Superoxide (O$_2^-$) mediated cell survival is NHE-1-dependant**

Recent evidence has suggested that increased intracellular O$_2^-$ anion promotes cell survival (Clement MV and Stemenkovic I, 1996; Pervaiz S et al, 1999), whether achieved by its overproduction or due to inhibition of the O$_2^-$ scavanger enzyme Copper/Zinc Superoxide Dismutase (Cu/Zn SOD). SOD catalyzes the conversion of O$_2^-$ to hydrogen peroxide (H$_2$O$_2$). This enzyme scavenges O$_2^-$ radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen:

$$2O_2^* + 2H^+ \xrightleftharpoons{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Diethyldithiocarbamate (DDC) is a selective inhibitor of Cu/Zn SOD. It has been used as an effective tool to increase intracellular O$_2^-$ levels by preventing its dismutation to H$_2$O$_2$ (Clement MV and Stemenkovic I, 1996). With the previous observations that O$_2^-$ acts as signal for cell survival and the results presented above we asked a simple question: could NHE-1 gene transcription be one of the targets responsible for O$_2^-$-
Figure 6. Silencing of NHE-1 gene leads to increased susceptibility to cell death in LNCaP cells.
LNCaP cells were transfected with control (Co) or NHE1 siRNA (NHE1). Forty-eight hours post-silencing (A) western blot analysis was done and (B) cell death was triggered for 18 hours with either 0.5 μM staurosporine (Sts 0.5) or 20 μM etoposide (Eto 20) before being assessed using the β-gal assay as described in Materials and Methods. Results are shown as mean of 3 experiments done in duplicate ± SE. * P < 0.02

Figure 7. Caspase 3 (DEVDase) activity in NHE-1 silenced LNCaP cells upon treatment with etoposide and staurosporine.
LNCaP cells were transfected with either Control (Co) or NHE1 siRNA (NHE1). Forty-eight hours post-silencing cell death was triggered with either 0.5 μM staurosporine (Sts 0.5) or 20 μM etoposide (Eto 20). Lysates were prepared at 4 (4H) and 8 hours (8H) and DEVDase activity was measured. Results show mean of 3 experiments done in duplicate ± SE. * P < 0.02
mediated inhibition of cell death. To test this hypothesis, NIH3T3 cells were transfected either with a control or NHE1 siRNA. Fig 8 is a control experiment to show that NHE-1 siRNA or cDNA transfection on its own doesn’t affect the intracellular $O_2^-$ levels. In NIH3T3 cells, twenty-four hours post transfection, serum was reduced to 0.5% for 24 hours prior to incubation with or without 1 mM DDC. In one set of cells 10% serum was added and $O_2^-$ levels were assessed and this was used as a positive control. As shown in Fig 9 DDC brings up $O_2^-$ in serum-starved cells almost up to the level comparable to cells grown in 10% serum. DDC was able to elicit an increase in intracellular $O_2^-$ levels in both control and NHE1 silenced cells, thus the ability of DDC to increase $O_2^-$ levels is independent of the level of NHE-1 expression. After incubation with DDC for 10 hours, when cell death was induced using 0.5 µM staurosporine for 18 hours, it was observed that decreased NHE1 expression prevented DDC mediated inhibition of staurosporine-induced cell death (Fig 10). In this set of experiments, crystal violet assay was used to assess cell death, as the transfection efficiency obtained with siRNA was routinely around 90%.

To confirm that the type of cell death was apoptotic, Caspase 3 activity was assessed in the same experimental setting. Firstly caspase 3 activity was measured at 2, 4, 8 and 12 hours time points. Staurosporine in the concentration of 0.5 µM does not kill NIH3T3 cells very quickly; rather there are around 30% viable cells even after 18 hours incubation with staurosporine (Fig 10B). In NHE-1 silenced NIH3T3 cells caspase 3 activity was higher at 4 and 8 hour time points as compared to control cells, but this difference was abrogated after longer incubation with staurosporine (Fig 10C).
Figure 8. Manipulation of NHE-1 expression does not affect intracellular superoxide levels.
NIH3T3 cells were transfected with either pIRES, NHE1 cDNA (pECE-NHE1), control siRNA (Co siRNA) or NHE1 siRNA. Forty eight hours post-transfection, intracellular superoxide levels were assessed by lucigenin assay as described in Materials and Methods. Results are shown as percent increase against non-transfected cells left in normal medium. Results show mean of 2 experiments done in triplicate.

Figure 9. DDC leads to significant increase in intracellular superoxide levels in NIH3T3 cells.
NIH3T3 cells were transfected with either control (Co) or NHE-1siRNA (NHE1) as described in Materials and Methods. Twenty-four hours post-transfection, NIH3T3 cells were incubated in medium with 0.5% serum for another 24 hours prior incubation with or without 1mM DDC for 2 hours or with 10% serum, before measurement of intracellular superoxide. Superoxide level was assessed using lucigenein-based assay as described in Materials and Methods. Results show mean of 3 experiments done in triplicate ± SE. * P < 0.02
On careful analysis of data in Fig 10D it can be seen that control cells treated with DDC show the lowest activation of caspase 3 and NHE-1 silenced cells without DDC treatment show the highest.

Taken together, these data demonstrate that DDC mediated inhibition of staurosporine-induced cell death in NIH3T3 cells is dependent on NHE-1 protein expression. Interestingly, it can be noted in Fig 10A that DDC treatment for at least 10 hours led to an increase in NHE1 protein expression, more so in control cells. Lack of this effect in NHE1-silenced cells can be due to the effectiveness of NHE1 siRNA in blocking translation of this protein. Thus, in summary, incubation of NIH3T3 cells with DDC resulted in an increased inhibition of cell response to death triggers that was dependent on NHE-1 expression.

**III.2.b Role of superoxide (O$_2^-$) in NHE1-dependent cell survival in tumor cells**

Pro-oxidant state or increase in intracellular level of reactive oxygen species such as O$_2^-$ is a characteristic of tumor cells (Cerutti PA, 1985). To investigate the proposed role of O$_2^-$ in regulation of NHE-1, U87 cells were incubated either in 0.5% serum, 10% serum or 0.5% serum plus diphenylene iodonium (DPI). DPI is an NADPH oxidase inhibitor and has been used widely to inhibit intracellular O$_2^-$ levels. As shown in Fig 11A treatment with 6 μM DPI for 24 hours inhibits serum-induced production of O$_2^-$, and it even brings the level lower then serum-starved cells. Indeed, this can be explained on the basis that U87 cells are highly malignant and tumor cells usually display an altered redox status, and thus addition of DPI shows a significant drop in intracellular levels of O$_2^-$ levels. Interestingly and as expected, DPI-induced decrease in O$_2^-$ was translated to a decrease in NHE-1 protein expression in U87 cells.
Figure 10. DDC-mediated inhibition of cell death is dependent on NHE-1 gene expression. (continued on next page)
Figure 10. DDC-mediated inhibition of cell death is dependent on NHE-1 gene expression.

NIH3T3 cells were transfected with either control (Co) or NHE-1 siRNA (NHE1) as described in Materials and Methods. Twenty-four hours post-transfection, NIH3T3 cells were incubated in medium with 0.5% serum for another 24 hours prior incubation with or without 1mM DDC for 10 hours before (A) Western blot analysis was done. 0.5 μM staurosporine (Sts) was then added for (B) 18 hours before being assessed for cell death. Cell death was assessed using crystal violet assay as described in Materials and methods. Cell death was calculated as (OD value of cells incubated with staurosporine / OD value of cells left for the same period of time without staurosporine). (C) Caspase assay was performed at 0, 2, 4, 8 and 12 hours after adding staurosporine. (D) Eight-hours time point was chosen for caspase 3 activity assays in subsequent experiments. Results show mean of 3 experiments done in duplicate ± SE. * P < 0.02
(Fig 11B). Multiple bands seen in the western blot analysis of NHE-1 represent various levels of maturity of NHE-1 protein, as glycosylation is the major post-translational modification that takes place as the NHE-1 protein migrates to the plasma membrane (Bullis et al, 2002). Fully glycosylated form of NHE-1 protein has a molecular weight of approximately 105 kD.

To investigate further that the $\mathrm{O}_2^-$-dependent increase in NHE1 expression leads to cell survival, U87 cells were either transfected with NHE1 cDNA or Control and NHE1 siRNA. As U87 cells growth rate is much faster as compared to NIH3T3 cells, all triggers had to be added 24 hours post-transfection in contrast to the usual 48 hours, so that the cells didn’t become over-confluent. NHE1 protein was either over-expressed or silenced using NHE1 cDNA and siRNA respectively to achieve varying levels of NHE1 protein expression. Twenty-four hours post transfection cells were treated with or without 6 $\mu$M DPI for 10 hours. Western blot analysis (Fig 12A) shows that a range of NHE1 expression was achieved across the spectrum of these transfection conditions. Addition of DPI reduced the level of expression of NHE1 in all transfection conditions, but less so in NHE-1 cDNA transfected cells (Fig 12A). In NHE-1 cDNA (pECE-NHE1) transfection, only endogenous level of NHE1 should be affected by incubation with DPI, as the transfected NHE-1 is under its own constitutive promoter control. Following the incubation with DPI, 20 $\mu$M etoposide was added to the cells to induce apoptosis. Caspase 3 activity was assessed after 8 hours incubation with etoposide. Indeed it was noted that the lower the expression of NHE1 protein the higher was the caspase 3 activation (Fig 12B).

It has been demonstrated that decreasing intracellular level of $\mathrm{O}_2^-$ results in restoration of tumor cells’ sensitivity to cell death triggers (Pervaiz S and Clement MV, 2002 and 2004; Clement MV and Pervaiz S, 1999). Indeed, DPI-mediated down-
Figure 11. Inhibition of intracellular superoxide production prevents NHE-1 protein expression in U87 cells.

U87 cells were grown in RPMI medium containing 0.5%, 10% serum or 10% serum and 6 μM DPI (10% serum +DPI) for 24 hours before (A) intracellular superoxide level, and (B) NHE-1 protein level was assessed by western blot analysis. Intracellular O$_2^•$ is expressed as % of cell left in 0.5% serum (% control cells). For A the results shown are mean of 3 experiments done in duplicate ± SE. For B the result shown is a representative experiment out of 2. * $P < 0.02$
regulation of NHE-1 expression correlated with an increase in sensitivity of U87 cells to etoposide induced cell death in a manner similar to that obtained upon silencing NHE-1 expression with NHE-1siRNA (see Fig 4B). These data suggest that the pro-oxidant milieu of tumor cells (increase in constitutive levels of intracellular O$_2^-$) maintains a level of NHE-1 gene expression that accounts for the resistance of cells to death stimuli. Caspase 3 activity in these experimental settings also correlated nicely with the expression level of NHE-1. It was observed that caspase 3 activation was higher in magnitude and occurred earlier in NHE1 silenced cells as compared to cells expressing normal or basal level of NHE1.

Manipulation of NHE1 protein levels downstream of O$_2^-$-mediated NHE-1 gene activation was sufficient to alter U87 cells’ response to etoposide-induced cell death. Taken together data shown in Fig 11 supports that expression of NHE1 correlates with intracellular level of O$_2^-$. Moreover results from Fig 10 and 12 shows that O$_2^-$ mediated inhibition of apoptotic cell death in NIH3T3 and U87 cells is dependent upon expression of NHE1. Hence, we next investigated the regulation of NHE1 expression.

### III.3. NHE-1 gene expression is growth factor regulated

NHE1 is activated by growth factors (Pouyssegur J, 1985), phorbol esters, and protein kinase C (PKC) (Mitsuka M and Berk BC, 1991). Increased mRNA levels of NHE1 have been reported during cellular proliferation in intact tissues (Elsing C et al, 1994). Thus NHE1 is activated by a wide array of signals that cause cellular growth and proliferation. The mechanisms involved in long-term regulation of NHE1 are only
Figure 12. Inhibition of intracellular superoxide production prevents NHE-1 expression in U87 cells and increases their susceptibility to etoposide-induced cell death.

NIH3T3 cells were either transfected with control siRNA (Co siRNA), NHE1 siRNA or pECE-NHE1 (NHE1 cDNA). Forty-eight hours post-transfection cells were either treated with DPI (6 μM) or left in normal medium for 10 hours before (A) western blot analysis or (B) adding 20 μM etoposide (Eto). Caspase 3 assay was performed at 8 hours after adding etoposide. Results show mean of 3 experiments ± SE.

DPI= cells left in DPI alone; Eto= cells left in etoposide alone; DPI+Eto= cells pre-incubated with DPI before adding etoposide. * P < 0.02
recently been studied. Recent cloning of NHE1 gene has permitted more in-depth study of promoter activity. Serum is an absolute requirement for cells growing in culture and it’s a well-documented activator of NHE1 gene expression (Besson P at al, 1998).

“NIH3T3 1A8” and “NIH3T3 Luc” cell lines, which were obtained from Dr Larry Fleigel, have been stably transfected with a proximal 1.1 kb fragment of mouse NHE1 promoter/enhancer upstream of a luciferase gene (Fig. 13). Thus, any signal that leads to increased NHE1 promoter activity would also result in increased luciferase enzyme production and this enzyme can be quantitatively measured by luciferase assay (as described in Materials and Methods). Both the cell lines were normally grown in 10% serum condition. Serum was withdrawn (i.e., cells were left in 0% serum and 0.5% serum) for 24 hours, a significantly higher NHE1 promoter activity was observed in those cells where serum was re-introduced for another 24 hours compared to those left throughout in 0% or 0.5% serum (Fig 14A). 0.5% serum was chosen for serum withdrawal conditions, as complete absence of serum would lead to complete cell cycle arrest and cell death over a prolonged incubation period. NIH3T3 1A8 cells were chosen for further study, as these cells showed better and more persistent results over the NIH3T3 Luc cells.

PMA has been known to produce superoxide in the cell, which was also confirmed in the current experimental setting. PMA is also a well-documented signal for NHE1 activity and expression. NIH3T3 1A8 cells were grown in 0% or 10% serum for 24 hours before being subjected to PMA and EGF. Incidentally, in addition to the increase in NHE-1 promoter activity, the presence of serum is also a stimulus for intracellular generation of $O_2^-$ that could be inhibited by incubating cells with the oxidase inhibitor DPI. Interestingly, incubation of NIH3T3 1A8 cells with DPI also
Figure 13A. NIH3T3 1A8 cells stably transfected with proximal 1.1 kb fragment of NHE-1 promoter/enhancer upstream of a luciferase gene. (Besson et al. Am. J Physiol. 274: C831- C839, 1998)

Figure 13B. Basic Principle of Luciferase Reporter Assay.
Figure 14. NHE-1 gene expression is growth factor regulated. 
(A) NIH3T3 1A8 (1A8) and NIH3T3 Luc (Luc) cells were grown in MEM medium supplemented with 10% FBS in 6-well plates to a 70% confluency. Cells were then left either in 10% serum, 0.5% serum or no serum with or without 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours. Cell lysates were prepared and Luciferase assay was performed as described in Materials and Methods. (B) NIH3T3 1A8 cells left in either 10% serum or no serum were treated with either 100 nM phorbol 12-myristate 13-acetate (PMA), 6 uM diphenyleneiodonium (DPI), 100 ng/ml Epidermal growth factor (EGF), PMA+DPI or EGF+DPI for 24 hours. Cell lysates were prepared and Luciferase assay was performed. Results in both A and B show mean of 3 experiments ± SE.
resulted in a significant inhibition of NHE-1 promoter activity (Fig 14B). In addition, PMA treatment in the presence of DPI did not show an increase in NHE1 promoter activity, again supporting the idea that O$_2^\cdot$ is a signal for NHE1 gene transcription. On the contrary, DPI was unable to block the EGF-induced NHE-1 promoter activation. EGF might have an O$_2^\cdot$-independent mechanism for NHE1 gene activation or it could be that it has multiple downstream pathways.

### III.4. Intracellular O$_2^\cdot$ activates NHE-1 promoter activity

A well-documented activator of NHE-1 gene expression is the presence of serum in the cell culture medium (Besson P et al, 1998). Indeed, mouse fibroblast NIH3T3 1A8 grown in 10% serum showed higher NHE-1 promoter activity compared to the same cells grown in 0.5% serum for 18 hours (Fig 15A). Moreover, serum-dependent increase in NHE-1 promoter activity correlated with a higher level of NHE-1 protein compared to serum-starved cells (Fig 15B). As discussed previously, in addition to an increase in NHE-1 promoter activity, the presence of serum is also a stimulus for the intracellular generation of O$_2^\cdot$ that could be partially inhibited by DPI (Fig 15C). Interestingly, incubation of NIH3T31A8 cells with DPI not only inhibited NHE-1 promoter activity, but also decreased NHE-1 protein expression to the level of cells grown in 0.5% serum (Fig 15B).

In order to confirm that O$_2^\cdot$ could be an activator of NHE-1 gene expression, intracellular O$_2^\cdot$ levels were manipulated in NIH3T3 1A8 cells and its effect on NHE1 promoter activation was studied. Increase in intracellular O$_2^\cdot$ in NIH3T3 1A8 cells was obtained either by pharmacological inhibition of Cu/Zn SOD with DDC or treatment with the herbicide paraquat (Fig 16). Paraquat is known to produce
Figure 15. Serum-induced activation of NHE-1 is dependant upon intracellular production of superoxide.

NIH3T3 1A8 cells were grown in MEM medium containing 0.5%, 10% serum or 10% serum and 6 µM DPI (10% serum +DPI) for 2 hours before C) level of O$_2^-$, or 24 hours before B) NHE-1 protein level, and A) NHE-1 promoter activity were measured as described in Materials and Methods. NHE-1 promoter activity and intracellular O$_2^-$ are expressed as % of cell left in 0.5% serum (% control cells). For B the result shown is a representative experiment out of 2. For A and C results are shown as mean of at least 3 experiments done in duplicate ± SE. * P < 0.02
superoxide in the cell through its redox cycling capability. Tiron was used as a selective $O_2^-$ scavenger. All these triggers were added in 0.5% serum condition in order to minimize the serum-induced production of $O_2^-$. NHE1 promoter activity and protein expression were then assessed (Fig 17A and B). Interestingly, all conditions that led to an increase in intracellular $O_2^-$ also led to an increased NHE1 gene transcription. In addition and as expected, the protein expression of NHE-1 was also significantly increased under these experimental conditions (Fig 17B). More importantly, pre-incubation of cells with the $O_2^-$ scavenger tiron reverted DDC-induced increases in intracellular $O_2^-$, NHE-1 promoter activity and protein expression (Fig 16, 17A and 17B).

III.5.a Small GTPase Rac1-mediated survival is dependent upon NHE-1 protein expression

In non-phagocytic cells, production of $O_2^-$ has long been attributed to mitochondrial respiration. However, during the 1990’s it became more and more evident that non-phagocytic cells could also produce low levels of $O_2^-$ through mechanisms similar to the one described for phagocytes. In particular, Sudaresan et al. showed production of intracellular $O_2^-$ upon growth factor-mediated activation of the small GTPases Rac1 in fibroblasts (Sundaresan M et al, 1996). Moreover Irani et al. clearly demonstrated the role for Rac1-mediated $O_2^-$ production in cell cycle regulation in tumor cells (Irani K et al, 1997 and 1998). In order to study the role of Rac1 activation, and its downstream production of $O_2^-$, in the induction of NHE-1 promoter activity, three cell lines were used. The mouse muscle L6 cells (Yang W et al, 1996) and the mouse renal fibroblast
Figure 16. Superoxide levels in NIH3T3 1A8 cells in response to different drugs.
NIH3T3 1A8 cells were incubated with MEM medium containing 0.5% FBS in order to reduce the background level of $\text{O}_2^-$ seen in cells grown in serum. Eighteen hours later either 250 uM paraquat, 1mM DDC, 1mM tiron or 1mM tiron plus 1mM DDC (Tiron+DDC) were added and $\text{O}_2^-$ level was assessed after 4 hours as described in Materials and Methods. In one set 10% serum was added and was used as a positive control. Results are shown as mean of at least 3 experiments done in duplicate ± SE. * $P < 0.02$
Figure 17. Superoxide is a signal for NHE-1 promoter activity.
NIH3T3 1A8 cells were incubated with MEM medium containing 0.5% FBS. Eighteen hours later either 250 μM paraquat, 1mM DDC, 1mM tiron or 1mM tiron plus 1mM DDC (Tiron+DDC) were added and 18 hours later A) NHE-1 promoter activity and B) NHE-1 protein level was assessed. In one set 10% serum was added and was used as a positive control. For A results are shown as mean of at least 3 experiments done in duplicate ± SE. For B the result shown is a representative gel out of 3 experiments. * P < 0.02
MCT cells, stably transfected with the 1.1-kb proximal fragment of the mouse NHE1 promoter/enhancer inserted 5′ to a luciferase gene (L6 1.1kb and MCT 1.1kb,) and NIH3T3 1A8 cells were transiently transfected with a constitutively active form of Rac1, RacV12. Luciferase activity of RacV12 transfected cells was compared to pIRES empty vector transfected cells. Indeed, a significant increase in NHE-1 promoter activity was observed upon RacV12 transfection in all three cell types (Fig 18A). Similar results where obtained with NIH3T3CatC2 and NIH3T3CatB3 cells (Fig 18B). These cell lines have been stably transfected with proximal 1.1 kb fragment of mouse NHE1 promoter upstream of a CAT sequence (these cells were also obtained from Dr Larry Fliegel). Reason for using these cells was to make sure that the effect that we see is due to direct activation of NHE1 promoter and not due to any direct effect on luciferase protein itself. Thus, CAT reporter assay was used to rule out the possibility of this artifact. Hence, data obtained with two different reporter genes in different cell lines support that activation of Rac1 stimulated NHE-1 promoter activity.

III.5.b Rac1-mediated NHE-1 protein expression is a function of its ability to produce superoxide

Activation of Rac1, in addition to triggering intracellular production of O$_2^-$, has also been shown to regulate actin polymerization and Jun kinase (JNK) activation (Campbell SL et al, 1998). In order to assess that Rac1-mediated induction of NHE-1 gene promoter activity was a function of an increase in intracellular O$_2^-$, NIH3T3 1A8 cells were transfected with partial myc-tagged loss-of-function mutants of Rac1 containing specific amino acid substitutions in the activated RacV12 background (Pervaiz S et al, 2001). The RacV12L37 mutant activates JNK but is defective in
Figure 18. Expression of RacV12 induces NHE-1 promoter activity in a variety of cells.
A) MCT 1.1kb, L6 1.1kb and NIH3T3 1A8, and B) NIH3T3 CatB3 and NIH3T3 CatC2 cells were transiently transfected with pIRESRacV12 (RacV12) or the empty pIRES vector control as described in Materials and Methods. Fourty-eight hours post-transfection cells were serum deprived (0.5%serum) for 18 hours before A) luciferase activity or B) CAT expression were measured as described in Materials and Methods. Results are shown as mean of 3 independent transfections done in duplicate ± SE. * P < 0.02 ** P < 0.05
inducing actin polymerization, whereas the RacV12H40 mutant induces actin polymerization but is defective in JNK activation. However, both of these mutations still retain the ability to generate intracellular $O_2^{-}$ (Joneson T and Bar-Sagi D, 1998). In contrast, the critical role of histidine 103 (H103) and lysine 166 (K166) of Rac1 protein in $O_2^{-}$ production has been established (Toporik A et al, 1998). Thus, RacV12H103A and RacV12K166E mutants are deficient in $O_2^{-}$ production (Fig 19). This differential ability of the Rac mutants to produce $O_2^{-}$ in the cell was used to investigate whether NADPH interaction domain of Rac1 is important for its ability to induce NHE-1 gene activation. NIH3T3 1A8 cells were co-transfected with the pCMVβgal plasmid, which encodes for the β-galactosidase protein (β-gal) and either the pIRES control vector or pIRES-RacV12, pIRES-RacV12L37, pIRES-RacV12H40, pIRES-RacV12H103A or pIRES-RacV12K166E. Forty-eight hours post-transfection, cells were serum starved (0.5% serum) for 18 hours before assessing NHE-1 promoter activity. Cells were serum starved in order to prevent serum-induced activation of the promoter and background activity of the endogenous Rac. Co-transfection with the pCMVβgal plasmid was used to standardize transfection efficiency within the different constructs. It should be pointed out that the β-galactosidase activity for each transfection, and the level of expression of Rac mutants 48 hours post-transfection in NIH3T3 1A8 cells were not significantly different. Hence, the differences in the ability of different plasmids to induce NHE-1 promoter activity could not be attributed to differences in protein expression. In addition, Fig 20A confirms that transient transfection of NIH3T3 1A8 cells with pIRESRacV12, pIRESRacV12L37, pIRESRacV12H40 induced a significant increase in intracellular $O_2^{-}$ compared to vector control transfected cells.
Figure 19. Rac loss-of-function mutants.
RacV12 and other Rac loss-of-function mutants have been cloned in pIRES expression vector. RacK166 and RacH103 are deficient in generating O$_2^-$ in the cell. Shaded box denotes known function and “?” means unknown function.
Figure 20. NADPH oxidase interaction domain of Rac1 is required for Rac1-induced NHE-1 promoter activity.
NIH3T3 1A8 cells were transiently transfected with myc-tagged loss-of-function mutants of Rac1. Forty-eight hours post-transfection A) cells were serum starved (0.5% serum) for 18 hours before production of O$_2^-$ was measured as described in Materials and Methods. Results are expressed as % of O$_2^-$ increased above cells transfected with the pIRES vector control (% of control) and B) cells were serum starved (0.5% serum) for 18 hours before NHE-1 promoter activity was measured as described in Materials and Methods. Results are shown as % of cells transfected with the pIRES vector control (% of control). Results are shown as mean of at least 3 independent transfections done in duplicate $\pm$ SE. * $P < 0.02$
while a minimum induction was observed with pIRESRacV12H103A or pIRESRacV12K166E. Interestingly, Rac1-mediated production of intracellular O$_2^-$ correlated with the ability of Rac1 to increase NHE-1 promoter activity (Fig 20B). These results indicate that the effect of Rac1 on NHE-1 promoter activity and expression was not linked to JNK activation or actin polymerization. These findings, together with the results obtained with DDC and paraquat, strongly support that intracellular production of O$_2^-$ is responsible for Rac1-dependent increase in NHE-1 promoter activity.

Resistance to cell death in NIH3T3 cells also correlated with the ability of Rac1 to produce intracellular O$_2^-$. Fig 21 shows the response of Rac mutants-transfected NIH3T3 cells to etoposide-induced killing. Indeed, previous work from our lab has shown that Rac expression plays an important role in inhibition of apoptosis to a variety of apoptotic signals. $\beta$-gal survival assay was used to assess cell death in this set of experiments. NIH3T3 cells over expressing pIRES-RacV12, pIRES-RacV12H40 or pIRES-RacV12L37 were more resistant to cell death as compared to cells transfected with pIRES control vector or the Rac mutants deficient in O$_2^-$ production (pIRES-RacV12H103A and pIRES-RacV12K166E).

**III.5.c Serum-induced NHE-1 expression might involve activation of Rac1**

Furthermore, we asked if serum-induced activation of NHE-1 promoter activity in NIH3T3 1A8 cells could be linked to activation of Rac1 through intracellular O$_2^-$ production. Serum induces intracellular production of O$_2^-$ and it also leads to activation of Rac1. In view of our previous findings that Rac1-induced O$_2^-$ production is a signal for NHE-1 gene expression, its plausible to propose that serum might
Figure 21. Rac1-mediated cell survival is dependant on its ability to produce superoxide.
NIH3T3 1A8 cells were transiently transfected with myc-tagged loss-of-function mutants of Rac1. Forty-eight hours post-transfection cells were incubated with or without etoposide for 18 hours and survival was assessed by β-Gal assay as described in Materials and Methods. Results are shown as % of cells transfected with the pIRES vector control (% of control). Results are shown as mean of at least 3 independent transfections done in duplicate ± SE. * P < 0.02
Figure 22. Expression of RacN17 inhibits serum-induced NHE-1 promoter activity.
NIH3T3 1A8 cells were transiently transfected with a control vector (pIRES) or a vector encoding a myc-tagged dominant negative form of Rac1 (pIRESRacN17). Forty-eight hours post-transfection, cells were serum starved (0.5% serum) for 18 hours before, serum was re-introduced to one set of cells for another 18 hours (A). (B) Expression of the myc-tagged RacN17 protein was detected by Western blot analysis as described in Materials and Methods. (C) Activation of NHE-1 promoter activity with serum in the presence (RacN17/serum) or absence of RacN17 (pIRES/serum). pIRES/no serum and RacN17/no serum represent transfected cells left in medium without serum. NHE-1 promoter activity was measured as described in Materials and Methods. Results are shown as Mean ± S.E of at least 3 independent transfections done in duplicate. * P < 0.02
require activation of Rac1 leading to increase in O$_2^-$ and consequently increased NHE-1 expression.

NIH3T3 1A8 cells were transiently transfected with the dominant negative allele of Rac1, RacN17 and 48 hours post-transfection cells were serum starved for 18 hours before inducing NHE-1 promoter activity by adding 10% serum in the culture medium (Fig 22). Indeed, similar to the effect of DPI, expression of RacN17 inhibited serum-induced production of intracellular O$_2^-$ and NHE-1 promoter activity. These data further support a link between Rac1-induced intracellular O$_2^-$ production and NHE-1 promoter activity and gene expression, and suggest that Rac activity might be important in serum-mediated induction of NHE1 transcription.

M14 human melanoma cells stably transfected with constitutively active form of Rac1 (RacV12) and those transfected with the empty pIRES vector were used to further address the role of Rac1. Indeed, previous studies have demonstrated that M14RacV12 cells display decreased cell sensitivity to etoposide-induced death compared to cells expressing the pIRES vector control (Pervaiz S et al, 2001). However, upon silencing NHE-1 protein (Fig 23), cells’ sensitivity to etoposide in M14RacV12 cells was brought to the level of M14pIRES cells transfected with the control siRNA (Fig 24). Interestingly, down regulation of NHE-1 expression in M14pIRES cells further increased M14 cells’ sensitivity to etoposide-mediated death. These results are in agreement with the results obtained with over-expression or silencing of NHE-1 in NIH3T3 cells and support that increased NHE-1 protein level is involved in Rac1/O$_2^-$-mediated cell survival. Rac1-mediated production of intracellular O$_2^-$ always correlated with the ability of Rac1 to increase NHE-1 promoter activity but was not linked to JNK activation or actin polymerization. These
Figure 23. Manipulation of NHE-1 protein expression in M14pIRES and M14pIRES-RacV12 cells by NHE-1 siRNA transfection. M14pIRES and M14RacV12 cells were transfected with an NHE-1 specific siRNA (NHE-1 siRNA) or a control siRNA (co siRNA). Fourty-eight hours post-transfection, (A) Western blot analysis was done. (B) shows average band intensity of NHE-1/β actin. Result shown in A is a representative gel out of 3. B represent average of 3 experiments ± S.E. * P < 0.02
Figure 24. Rac-induced cell survival is dependant upon NHE-1 expression.
M14pIRES and M14RacV12 cells were transfected with either control (Co siRNA) or NHE1 siRNA and 0.5µg pCMV-βgal as described in Materials and Methods. Forty-eight hours post-transfection, cell death was induced with 40 µM etoposide for 18 hours before being assessed by β-gal assay. Control cells represent M14pIRES cells transfected with the control siRNA. Cell death was determined by the β-Gal assay as described in Materials and Methods. Results are shown as Mean ± S.E of at least 3 independent transfections done in duplicate. * P < 0.02
findings, together with the results obtained with DDC, strongly support that activation
of NHE-1 gene promoter is a critical target for $O_2^-$-mediated survival.

III.6. $H_2O_2$ inhibits NHE-1 promoter activity

The intracellular concentration of $O_2^-$ in eukaryotic cells is tightly regulated by the
activity of the enzyme Cu/Zn SOD, which catalyzes the dismutation of $O_2^-$ to $H_2O_2$
and $O_2$. Therefore the first question that comes to mind is that whether the increase in
NHE-1 promoter activity was a direct effect of $O_2^-$ on NHE-1 gene transcription or a
result of $H_2O_2$ production leading to $H_2O_2$-dependent transcription of the NHE-1
gene. It was also important to rule this possibility out as redox-regulated transcription
factors like NFκB and AP-1 have been shown to be activated by $H_2O_2$ (Bowie A and
O’Neil LA, 2000). To test that, NIH3T3 1A8, cells were incubated with increasing
concentrations of $H_2O_2$ for 18H before the activity of NHE-1 promoter was assessed.
It should be pointed out that the concentration of $H_2O_2$ used throughout this study was
$\leq 150\mu M$ because higher concentrations elicited cytotoxicity. Interestingly, in
complete contrast to the stimulatory effect of $O_2^-$, results clearly showed a
concentration dependent repression of NHE-1 promoter activity by $H_2O_2$ (Fig 25A).
These results were corroborated by western blot analysis of NHE-1 protein in lysates
obtained from NIH3T3 1A8 cells treated for 18H with $H_2O_2$ (Fig 25C). Similar results
were obtained with NIH3T3 CatC2 and NIH3T3 CatB3 (two different clones of
NIH3T3 cells stably transfected with a 1.1-kb proximal fragment of the mouse NHE1
promoter/enhancer inserted 5’ to the chloremphenicol acetyl transferase (CAT) gene)
(Besson P et al, 1998) (Fig 25B). Non-toxic concentration of $H_2O_2$ did not activate the
NHE-1 promoter. Instead, incubation of all three cell lines with increasing
Figure 25. H$_2$O$_2$ inhibits NHE-1 promoter activity in NIH3T3 cells.
A) NIH3T3 1A8, B) NIH3T3CatB3 and NIH3T3CatC2 cells were incubated with increasing concentrations of H$_2$O$_2$ for 18 hours before the NHE-1 promoter activity was measured by either A) the luciferase assay or B) detection of the CAT protein as described in Materials and Methods. C) shows western blot analysis of NIH3T3 1A8. Gel in C) is a representative gel out of 3. Results are shown as mean of at least 3 experiments done in duplicate ± S.E. * $P < 0.02$
concentration of H$_2$O$_2$ resulted in inhibition of NHE-1 promoter activity. Taken together, these data provide strong evidence to support that the expression of NHE-1 is redox regulated, with distinctly opposing effects of O$_2^-$ and H$_2$O$_2$ on its promoter activity and protein expression. Moreover, the use of two different reporter gene rules out the possibility that the observed inhibitory effect on NHE-1 promoter activity could have been attributed to the inhibitory effect of H$_2$O$_2$ on the reporter gene (luciferase or CAT) itself.

In addition, results showing that incubation with DDC resulted in a significant activation of the promoter also favor O$_2^-$ rather than H$_2$O$_2$ in the activation of the NHE-1 gene transcription, as DDC inhibits Cu/Zn SOD and prevents dismutation of O$_2^-$ to H$_2$O$_2$.

**III.7. H$_2$O$_2$ leads to decreased NHE-1 expression and increased susceptibility to cell death**

With the data obtained from previous experiments, it was becoming increasingly evident that level of expression of NHE1 protein was of critical importance in determining a cells’ response to death triggers. Thus, H$_2$O$_2$-mediated repression of NHE1 promoter and decreased protein expression should have the same effect. Interestingly and as expected, similar to the result obtained by silencing NHE-1, pre-incubation of NIH3T3 cells with 50 and 100 μM H$_2$O$_2$ for 18 hours sensitized NIH3T3 cells to etoposide-induced cell death (Fig 26). As is clearly evident in the figure, H$_2$O$_2$ on its own did not cause cell death but its presence enhanced etoposide-induced cell death in NIH3T3 cells. It can be deduced that a decrease in NHE1 protein expression, independent of whether achieved by gene silencing, incubation with DPI or H$_2$O$_2$ has the same final effect, that is, increased sensitivity to death triggers.
Figure 26. H$_2$O$_2$ treatment of NIH3T3 cells results in increased susceptibility to etoposide-induced killing.

NIH3T3 cells were incubated for 18 hours with 50 or 100 µM H$_2$O$_2$ before cell death was triggered with 20 (Eto 20µM) or 40 µM etoposide (Eto 40µM). Control represents cells left in normal medium. Cell viability was assessed using the crystal violet assay and result is shown as OD at 595 nm. Results are shown as mean of at least 3 experiments done in duplicate ± S.E. * $P < 0.02$
Most studies on NHE1 have been focused on the regulation of its activity. The data presented here addresses a potentially very important mechanism of its gene regulation.

The data presented until now clearly shows that NHE1 gene is redox regulated, being upregulated by increased $\text{O}_2^-$ in the cell and repressed by $\text{H}_2\text{O}_2$. And that this gene regulation translates well into protein expression and affects cells’ response to death triggers. These findings were tested across a wide range of murine and human cell lines. As discussed earlier, a pro-oxidant intracellular milieu confers a permissive environment for the growth and proliferation of the cells. Regulation of NHE1 by $\text{O}_2^-$ could now be proposed as a mechanism underlying this pro-oxidant state that leads to tumor cell survival.

III.8. Regulation of intracellular pH as one of the mechanisms of NHE-1-mediated cell survival

The predominant cellular function of NHE-1 is the regulation of intracellular pH (pHi). In addition to protecting cells from intracellular acidification, NHE has also been shown to initiate shifts in pH that stimulate growth of cells and plays a role in malignant transformation (Reshkin SJ et al, 2000). However, it is widely accepted that at physiological pH the exchanger is virtually in a quiescent state in unstimulated cells. Hence, increase in NHE-1 protein level in NIH3T3 may not be expected to significantly affect steady state pH. Indeed, over-expression of NHE-1 in NIH3T3 cells transiently transfected with 5μg of pECE-NHE1 resulted in 40% increase in NHE-1 protein level (see Fig 1A), but had a very minimal effect on pH (from 7.22±0.01 in the vector control transfected cells to 7.29±0.01 in cells over-expressing NHE-1) (Fig 27).
**Figure 27. Increased NHE1 protein expression level leads to a minimal increase in pHi.**

NIH3T3 cells were transfected with increasing concentrations of the pECE vector encoding for the human NHE-1 cDNA, and empty vector pIRES was added corresponding to varying pECE-NHE1 cDNA in order to keep the total cDNA amount constant. Forty-eight hours post transfection intracellular pH was measured using BCECF assay as described in Materials and Methods. Results are shown as mean of at least 3 experiments done in duplicate ± S.E. * $P < 0.02$

**Figure 28. Silencing of NHE1 gene results in a minimal decrease in intracellular pH in NIH3T3 cells.**

NIH3T3 cells were either transfected with NHE1 siRNA or control siRNA (Co siRNA). Forty-eight hours post transfection intracellular pH was measured using BCECF assay. Results are shown as mean of at least 3 experiments done in duplicate ± S.E. * $P < 0.02$
On the contrary, upon silencing of NHE-1 gene, a drop in intracellular pH was observed but again, the difference was very subtle with ∆pH value of only 0.07 units (Fig 28). Upon transfection of NIH3T3 cells with RacV12 and other Rac-loss-of-function mutants, the effect of these mutants on the NHE-1 promoter and O$_2^-$ inducing activity (see Fig 21) correlated with their effect on cytosolic pH; RacV12, RacH40 and RacL37 induced cytosolic alkalinization, whereas the two mutants lacking O$_2^-$ inducing activity of Rac1 (RacK166 and RacH103) failed to alter cytosolic pH (Figure 29).

On the other hand, when M14pIRES and M14RacV12 cells were transfected with NHE1 siRNA, there was a significant difference in terms of ∆pH value. As shown in Fig 30, the resting pH$_i$ values differ among M14pIRES and M14RacV12, with the later having a higher pH$_i$ value. Interestingly, different NHE-1 expression levels in M14 cells resulted in significant differences in pH$_i$. For example, the ∆pH between M14pIRES transfected with control siRNA and M14pIRES cells transfected with NHE1 siRNA was 0.25 units (Fig 30).

As discussed earlier, malignant gliomas are comprised of highly proliferating cancer cells, and exhibit increased glucose uptake and anaerobic glycolysis. Increased production of lactic acid leads to increased generation of H$^+$. Studies have indicated that malignant gliomas display a higher pH as compared to non-transformed astrocytes and this alkaline pH$_i$ has been attributed to the increased activity of NHE1 (Lee AM et al, 2000). Indeed, in U87 cells, comparing Co siRNA and NHE1 siRNA transfected cells, there was a significant decrease in resting pH$_i$, ∆pH of 0.27 units (Fig 31A). Upon exposure of cells to an acid load and then looking at the pH$_i$ recovery in a bicarbonate-free buffer, it could be confirmed that in NHE1-silenced increase in NHE-1 protein seems unlikely to play a critical role in regulating steady
state pHi in normal cells. NHE-1 pump activity rather than expression seems to be responsible for the pHi modulating function of this protein.

<table>
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<tr>
<th>pHi</th>
<th>6.94</th>
<th>7.08</th>
<th>6.99</th>
<th>6.93</th>
<th>7.04</th>
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<td>Rac K166</td>
<td>Rac H103</td>
<td>Rac H40</td>
<td>Rac L37</td>
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Figure 29. Increase in intracellular pH correlates with the ability of Rac mutants to produce superoxide and induce NHE1 transcription. NIH3T3 cells were transiently transfected with myc-tagged loss-of-function mutants of Rac1. Forty-eight hours post-transfection measurement of intracellular pH was done using BCECF assay as described in Materials and Methods. Results are shown as mean of at least 3 experiments done in duplicate + SE. * P < 0.02

Figure 30. Silencing of NHE1 gene results in a drop in intracellular pH in M14pIRES and M14RacV12 cells. M14pIRES and M14RacV12 cells were either transfected with NHE1 siRNA or Co siRNA. Forty-eight hours post transfection intracellular pH was measured using BCECF assay. Results are shown as mean of at least 3 experiments done in duplicate + SE. * P < 0.02
Figure 31A. Silencing of NHE1 gene results in a drop in intracellular pH in U87 cells.
U87 cells were either transfected with NHE1 siRNA or Co siRNA. Twenty-four hours post transfection intracellular pH was measured using BCECF assay. Results are shown as mean of at least 2 experiments done in duplicate + SE. * P < 0.02

Figure 31B. Decreased expression of NHE1 leads to decreased activity of the pump in U87 cells.
U87 cells were either transfected with NHE1 siRNA or Co siRNA. Twenty-four hours post transfection intracellular pH recovery assay was performed as described in Materials and Methods. Results show mean of 2 experiments done in triplicate.
U87 cells there was a blunted recovery phase (Fig 31B) as compared to the control cells.

Hence, taken together the data from NIH3T3, M14 and U87 cells, O$_2^-$-mediated state pHi in normal cells. NHE-1 pump activity rather then expression seems to be responsible for the pHi modulating function of this protein.

Pharmacological inhibition of NHE1 activity has been achieved with the diuretic amiloride and its derivatives. Although the exact mechanism is not yet known, NHE1 inhibitors are thought to act by competitively inhibiting Na$^+$ binding at the extracellular cation-binding site (Putney LK et al, 2000). Indeed, transient transfection of NIH3T3 cells with RacV12 resulted in an increase in pHi that could be reverted by incubation with amiloride (Figure 32). Similar results were obtained with M14 cells. M14RacV12 cells had a higher resting pHi than the vector transfected cells that could be reverted following inhibition of NHE-1 activity with amiloride (Figure 33). These data indicate that the increase in resting pHi induced upon transfection (of NIH3T3 cells or M14 melanoma cells) with RacV12 is dependent on the activity of the NHE-1 exchanger.

### III.9. Region of NHE-1 promoter involved in O$_2^-$-mediated activation

Some preliminary work was done in order to delineate the region of NHE1 promoter that responds to O$_2^-$-mediated activation. L6 rat myoblasts stably transfected with different truncated versions of NHE-1 promoter/enhancer upstream of a luciferase gene were used (Yang W et al, 1996). These cells were also obtained from Dr Larry Fliegel’s lab. Fig 34A shows a schematic diagram of the constructs used for transfection of L6 muscle cells. Reporter constructs terminated at bps -808, -515 -171,
**Fig 32. Increased intracellular pH in RacV12 over-expressing NIH3T3 cells is dependent upon NHE1 activity.**

NIH3T3 cells were transiently transfected with pIRESRacV12 or empty vector pIRES. Forty-eight hours post-transfection A) cells were treated with or without 500 uM Amiloride for another 24 hours. Intracellular pH was measured by BCECF assay. Results are shown as mean of at least 3 independent transfections done in duplicate ± S.E. *P < 0.02

**Fig 33. Increased intracellular pH in M14 cells is a function of NHE1 activity.**

M14pIRES and M14RacV12 cells were grown with or without Amiloride (500 uM) for 24 hours before intracellular pH was measured using BCECF assay. Results are shown as mean of at least 3 independent transfections done in duplicate ± S.E *P < 0.02
Figure 34. NHE-1 promoter/enhancer constructs.
(A) Different length truncated NHE-1 promoter constructs stably transfected into L6 cells. (B) Comparison of mouse and human NHE-1 promoter.
-155, and -125 respectively for pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP, and pMP +AP2.

Yang et al have demonstrated that deletion of the promoter up to and including the AP2 site resulted in almost total elimination of activity. Inclusion of the AP2 site (-125) resulted in a significant increase in activity of the promoter. Inclusion of base pairs - 155 to - 126 (pXP-0.18MP plasmid) resulted in a small insignificant increase in the activity of the promoter. However inclusion of 16 more base pairs including a pyrimidine rich region resulted in a large, significant increase in activity of the promoter. Inclusion of up to the entire 8.5 kb region of the NHE1 promoter did not result in further significant increases in activity of the promoter (Yang W et al, 1996). Thus, AP2 binding site and the pyrimidine-rich region of mouse promoter have been shown to play a significant role in gene regulation. Using DNA footprinting experiments Kolyada et al have suggested that there are 4 regions of the human NHE1 promoter (A-D) that may be important in NHE1 expression (Kolyada AY et al, 1994). Comparison of these regions in human NHE-1 to mouse NHE1 promoter is shown in Fig 34B.

As we were interested in narrowing down the region of NHE-1 promoter that responds to O$_2^-$-mediated activation, L6 cells were incubated with herbicide paraquat to increase intracellular O$_2^-$ levels. First a non-toxic dose of paraquat was determined that led to a significant rise in intracellular levels of O$_2^-$ (Fig 35).

When L6/1.1kb, L6/0.9kb, L6/0.5kb, L6/0.2kb and L6/0.18kb were incubated with 125 μM paraquat, it lead to an increase in intracellular O$_2^-$ levels (Fig 36A). When NHE-1 promoter activity was measured in these cells, it was noted that L6 cells transfected with 1.1, 0.9, 0.5 and 0.2kb fragments of NHE-1 promoter showed an
Figure 35. Low dose of paraquat leads to increased superoxide production in L6 cells.

L6/1.1kb cells were incubated with varying concentrations of herbicide paraquat for 4 hours. Lucigenin assay was performed as described in Materials and Methods. Results show mean of 2 experiments done in duplicate.
Figure 36. Superoxide-mediated NHE1 gene transcription in L6 cells.
L6/1.1, L6/0.9, L6/0.5, L6/0.2 and L6/0.18kb cells were incubated with 0.125 mM paraquat. (A) Lucigenin assay was performed 2 hours after addition of paraquat and (B) Luciferase assay was performed at 10 hours as described in Materials and Methods. Results show mean of 2 experiments done in duplicate.
increaser in promoter activity, while further reduction of promoter length resulted in low or no response (Fig 36B).

To confirm these findings, L6/1.1kb, L6/0.5kb, L6/0.18kb and L6/AP2+ cells were transfected either with pIRESRacV12 or empty pIRES vector (Fig 37). Fourty eight hours post-transfection, NHE-1 promoter activity was measured and interestingly it complemented the data obtained with paraquat treatment of L6 cells. Thus we can conclude here that $O_2^-$-mediated activation of NHE-1 promoter lies upstream than the AP2-binding site, and somewhere between the 0.18kb and 0.5kb region of the promoter. However these data are preliminary results that require extensive research work in order to narrow down the $O_2^-$-responsive element on NHE-1 promoter or before a candidate transcription factor(s) could be proposed to act downstream of $O_2^-$ generation.
Figure 37. Rac1-induced transcription of NHE-1 is not seen below 0.5 kb in L6 cells.
L6/1.1, L6/0.5, L6/0.18 and L6AP2+ cells were transiently transfected with pIRES empty vector or pIRESRacV12 cDNA. Forty eight hours post-transfection, luciferase assay was performed as mentioned in Materials and Methods. Results are shown as percentage increase in luciferase activity in RacV12 transfected cells against the pIRES vector transfected cells. Results are shown as Mean ± S.E of at least 3 independent transfections done in duplicate.
Chapter IV
Discussion and conclusions

IV.1. How tumors develop

Normal tissue homeostasis requires the physiological deletion of cells by process of apoptosis, a genetically determined programmed cell death (Kerr JF et al, 1972). Taken together, apoptotic processes are of widespread biological significance, being involved in development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defective and therefore harmful cells. Thus, deregulation of the apoptotic program is implicated in a variety of pathological conditions: defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections; while on the other hand neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis (Fadeel et al., 1999).

Tumors arise because the homeostatic control mechanisms that maintain the appropriate number of cells in normal tissues is defective, leading to an imbalance between cell proliferation and cell death. This leads to an uncontrolled expansion of the cell population. Thus, one of the major challenges cancer research has faced in past decades has been to devise ways to induce apoptosis in tumor cells or make them more sensitive to apoptotic triggers.

IV.2. Permissive intracellular milieu

One of the mechanisms through which cancer cells evade death signals and proliferate unchecked is by maintaining a permissive intracellular environment. Slightly pro-
oxidant intracellular milieu is a characteristic many tumors share and it has been proposed to promote cell growth and inhibit cell death. It is established that a pro-oxidant state where intracellular concentrations of activated forms of oxygen are increased can modulate expression of early growth-related genes, leading in some cases to aberrant cell growth and differentiation (Burdon et al., 1995). Indeed, increased metabolic rate and ROS production in tumor cells may provide them with a survival advantage over their normal counterparts (Cerutti PA, 1985). The field of ROS research has witnessed a revolution of ideas. Highly reactive and damaging ROS are now thought to play a role in a variety of physiological functions (Finkel T, 1998 and 2000). Interestingly, the regulation of tumor cells’ sensitivity to death stimuli has been shown to be linked to the intracellular levels of $O_2^-$ and $H_2O_2$ (Pervaiz S and Clement MV, 2002 and 2004; Clement MV and Pervaiz S, 1999 and 2001). Classically, the role of ROS in inducing necrotic cell death is well accepted, however the role of ROS in apoptosis is more controversial. Though a fair amount of controversy exists with respect to the precise pro- or anti-apoptotic functions of $O_2^-$ and $H_2O_2$, some inducers of apoptosis stimulate intracellular production of $H_2O_2$, and many inhibitors of apoptosis have antioxidant properties or enhance the cellular antioxidant defense mechanism. These observations have led to the suggestion that ROS are effectors for a variety of triggers of apoptosis (Clement MV and Pervaiz S, 2001).

IV.3. Alkaline pHi and role of NHE-1

In addition to a pro-oxidant state, tumor cells also display altered intracellular pH (pHi) regulation. Maintenance of intracellular pH within a very tight range is important for most if not all physiological functions. It has been shown that cellular
pH is crucial for biological functions such as cell proliferation, invasion and metastasis, drug resistance and apoptosis. Intracellular acidification has been shown to be a trigger in the early phase of apoptosis and leads to activation of endonucleases inducing DNA fragmentation. To avoid intracellular acidification under such conditions, pH regulators are thought to be up-regulated in tumour cells. Indeed, it was recently demonstrated that pre-treating tumor cells with proton pump inhibitors (PPIs) significantly increased their sensitivity to killing by conventional chemotherapeutic drugs (Francesca et al., 2004). It has been proposed that regulation of intracellular pH may be a possible mechanism for tumour-selective therapy (Raghunand et al., 1999; Wong P et al, 2002; Wahl ML et al, 2002). The past decade has also seen the discovery of the physiological mechanism by which a decrease in intracellular pH is the initial trigger for a cascade of events resulting in apoptosis. Multiple Drug Resistance (MDR) phenotype of tumor cells has also been linked to an alkaline pH in tumor cells (Keizer HG and Joenje H, 1989; Weinsburg JH et al, 1999).

The ubiquitously expressed Na\(^+\)-H\(^+\) exchanger NHE-1 is one of several ion exchangers involved in pH homeostasis. Although NHE-1’s role in tumorigenesis has been long known, but recent advances in molecular understanding of its functions and cloning of NHE-1 gene has shed a new light on this potentially very important subject. Evidence strongly suggests that, in addition to regulating pH NHE-1 could be a critical regulator of cell sensitivity to death stimuli (Wu KL et al, 2003) and that this ability of NHE-1 may go well beyond its primary function of pH homeostasis. NHE-1 has also been shown not only to facilitate tumor development but also to maintain tumorigenic phenotype (Reshkin SJ et al, 2000).
IV.4. Regulation of NHE-1

NHE-1 could be regulated at multiple levels. Although the increase in transport activity of NHE-1 is often associated with phosphorylation of a number of serine residues within the distal C-terminal cytoplasmic domain of the NHE-1 protein, there is strong evidence that NHE-1 activity is regulated by phosphorylation dependent as well as independent mechanisms (Putney LK et al, 2002). Many transcription factors that are important during cell development and differentiation have also been shown to activate NHE1 gene. Evidence suggests that NHE1 activation at both transcription and protein level plays an important role in regulating its function. Mitogenic stimulation of NHE1 leads to increased gene transcription and increased expression of NHE1 protein on cell surface (Besson P et al, 1998). Furthermore, there is evidence to support that mRNA levels of the exchanger are increased during cellular proliferation in intact tissues (Elsing C et al, 1994).

IV.5. Rationale of our study

Transcription of NHE-1 could be increased by a variety of stimuli, such as serum, acidosis, PKC and cell proliferation (Besson P et al, 1998; Putney LK et al, 2002); however the signals involved in NHE-1 gene transcription remain largely unknown. It is really intriguing to note that a slight pro-oxidant intracellular environment also shares the pro-survival and proliferative properties of NHE-1. Indeed it has been recently demonstrated that cellular redox status is important in maintaining an alkaline pH (Pervaiz S and Clement M V, 2002a). In addition, the link between intracellular ROS and NHE-1 is supported by the observations that the aforementioned inducers of NHE-1 gene expression have also been implicated in intracellular ROS production. Intrigued by these findings, we set out to investigate
whether there exists a true cause-and-effect relationship between the pro-survival activity of O$_2^-$ and NHE-1.

**IV.6. Regulation of NHE-1 gene expression regulates cells’ response to death triggers**

It has been shown that mitogenic stimulation of NHE1 leads to increased gene transcription and increased expression of NHE1 protein on cell surface (Besson P et al, 1998). Therefore we first asked if regulation of NHE-1 gene expression affected cell sensitivity to apoptosis. We show clearly that level of NHE-1 gene expression regulates the sensitivity of mouse fibroblast NIH3T3 cells, the human glioma U87 cells and the human melanoma M14 cells, to etoposide- or staurosporine-induced cell death. NHE-1 was either over-expressed in these cells or silenced by using a specific siRNA, and a range of protein expression level was achieved. Cell death assessment in these experiments was done using the β-gal assay as described in Materials and Methods and reported previously (Pervaiz S et al, 2001). This method was used because it allows for the measurement of cell death in transfected cells only. This approach is particularly important when over-expressing NHE-1 keeping in view the transfection efficiency of ~30% usually obtained in these experiments. Hence, measurement of cell death in β-gal transfected cells is a better index of the effect of NHE-1 over-expression on cells’ response to death triggers. On the contrary transfection of cells with the specific NHE-1 siRNA routinely affects 90% of the cells. However, in order to compare results obtained by over-expression or down-regulation of NHE-1 protein the β-gal assay was also used when assessing NIH3T3 cells’ response to staurosporine-induced cell death upon silencing of the NHE-1 gene expression. Hence, taken together, these data show that staurosporine can either
induce 15% or 48% death in NIH3T3 cells depending on the level of NHE-1 expression (Fig 3).

Gliomas and malignant melanomas are amongst the most aggressive human cancers. By using U87 and M14 cells, it was very encouraging to observe that by silencing the NHE-1 gene, there was a significant increase in their sensitivity to death triggers like staurosporine and etoposide. To confirm that death induced by etoposide and staurosporine was apoptotic in nature, caspase 3 activity was measured. It was noted that in NHE-1 silenced cells caspase 3 activation was induced earlier and the magnitude was higher as compared to control cells. Although, this difference in caspase activity was abrogated when cells were incubated with etoposide or staurosporine for longer duration (i.e; 18-24 hours). The fact that there was a difference in the pattern of initial activation of caspase 3 makes more sense in physiological terms, as prolonged incubation with cytotoxic drugs will ultimately induce apoptosis or even necrosis. An ideal scenario is where lower doses of cytotoxic drugs would effectively kill tumor cells leaving normal cells virtually unharmed.

The relative differences observed in caspase activities were not very marked. This could be due to low level of caspase activation, or even some component of caspase-independent cell death. Despite a low level of caspase activation, differences in magnitude of cell death sensitivity between control and NHE1-silenced cells were significantly marked. Interestingly, a recent study has reported caspase-independent paraptosis like mode of cell death following NHE-1 inhibition (Schneider D et al, 2004). In summary, by manipulating the protein expression of NHE-1, we were able to alter cells’ sensitivity to apoptotic triggers.

IV.7. Superoxide (O$_2^-$) mediated cell survival is NHE-1-dependant
As described earlier, there is evidence to suggest that transformation and proliferation are favored during intracellular redox imbalance leading to a pro-oxidant intracellular milieu (Cerutti PA, 1985). The effect of $O_2^-$ on NHE-1-dependent survival was assessed by several experiments. Hence, to test if an increase in NHE-1 expression could be the mechanism involved in $O_2^-$-mediated survival, we tested the effect of NHE-1 gene silencing with a specific NHE-1 siRNA on the ability of DDC to increase intracellular $O_2^-$ and inhibit cell death induced by exposure to staurosporine. Silencing of NHE-1 gene expression did not prevent DDC-induced increase in intracellular level of $O_2^-$ in NIH3T3 cells, however, NHE-1siRNA prevented DDC-mediated inhibition of staurosporine-induced cell death. Secondly, serum-induced NHE-1 protein expression was partially inhibited by the widely used oxidase inhibitor, DPI; incubation of serum induced U87 cells with DPI resulted in inhibition of intracellular $O_2^-$ production and prevented the increase in NHE-1 protein synthesis. Taken together, these findings suggest that, in addition to activation of the exchanger leading to alkaline pH, redox regulation of NHE-1 gene expression could be critical in tumor cell formation. In particular, resistance of tumor cells to death triggers due to a slight increase in intracellular $O_2^-$ could be linked to the regulation of NHE-1 protein expression.

**IV.8. NHE-1 gene expression is growth factor regulated**

NHE-1 is activated by a wide variety of signals that promote cellular growth and proliferation. A well-documented activator of NHE-1 gene expression is the presence of serum in the cell culture medium (Besson P et al, 1998). Tumor cells usually are not dependent on growth factor stimulation and in cell culture are relatively independent to serum supplementation, while normal cells would die if not supplied
with serum. Activation of survival pathways in tumor cells are achieved by growth factor-independent pathways. The fact that NHE-1 is over-expressed and shows higher activity in tumor cells indicates an alternative pathway. Indeed the results obtained with dominant negative Rac1 (RacN17) indicate that serum-induced NHE-1 promoter activation is, at least in part, dependent on activation of Rac1 (discussed in detail later). Thus, activated Rac in tumor cells might act as a substitute to serum/growth factors.

IV.9. Intracellular \( \text{O}_2^- \) activates NHE-1 promoter activity

Mouse fibroblast NIH3T3 cells, stably transfected with the 1.1-kb proximal fragment of the mouse NHE1 promoter/enhancer inserted 5’to a luciferase gene (NIH3T3 1A8) (Besson P et al, 1998) were used to study the effect of serum and various drugs on NHE-1 promoter. In addition to NHE-1 promoter activation, serum is also known to produce superoxide in the cell. Indeed, generation of \( \text{O}_2^- \) could be partially inhibited by incubating cells with the widely used oxidase inhibitor DPI. Interestingly, incubation of NIH3T3 1A8 cells with DPI also resulted in the inhibition of NHE-1 promoter activity and decrease in NHE-1 protein expression to the level of cells grown in 0.5% serum. DDC-induced activation of NHE-1 promoter in the absence of serum also provides evidence in favor of \( \text{O}_2^- \) being an activator of NHE-1 gene expression. In addition, pre-incubation of cells with the \( \text{O}_2^- \) scavenger tiron reverted DDC-induced increases in intracellular \( \text{O}_2^- \), NHE-1 promoter activity and protein expression. Taken together these results strongly support a role for \( \text{O}_2^- \) production as a signaling molecule for NHE-1 gene expression, and show that serum-induced activation of NHE-1 involves \( \text{O}_2^- \). Moreover, as discussed previously, tumors display a pro-oxidant intracellular environment, it is very intriguing to note that a slight
increase in intracellular $O_2^-$ levels can lead to an increased firing of NHE-1 promoter and subsequently increased protein expression. As tumor cells display a chronic and persistent phase of proliferation and inhibition of cell death signals, it seems imperative that NHE1 has to be regulated on long-term basis if it were to confer the cells with a survival advantage. Thus a scenario of increased intracellular $O_2^-$ leading to a persistently increased baseline expression of NHE-1 in tumor cells could explain, in part, the inherent ability of these cells to resist normal apoptotic triggers.

IV.10. Small GTPase Rac1-mediated survival is dependent upon NHE-1 protein expression

Mutated Ras is an oncogene found in many human cancers. Rac is an important downstream target of Ras which has multiple functions in the cell. Interestingly, mitogenic signal triggered by the oncogene Ras in a fibroblast cell line has been attributed to Rac-dependent intracellular production of $O_2^-$, thereby, once again lending support to the hypothesis that $O_2^-$ acts as an important proliferative signal during tumorigenesis (Irani K et al, 1997). A role for heterotrimeric G protein subunits and low molecular weight GTPases in the stimulation of NHE-1 activity was first shown in 1996 when Hooley et al demonstrated that NHE-1 is a downstream effector of the Rho family of GTPase (Hooley R et al, 1996). However, activation of NHE-1 activity through these signaling pathways was mainly attributed to direct activation of kinases that induce NHE-1 phosphorylation, and Rac1-mediated activation of NHE-1 was shown to involve a MEKK1-dependent mechanism. However, in the light of our findings, in addition to the induction of a phosphorylation-dependent activation of NHE-1, Rac1 could also mediate increase in NHE-1 expression through transcriptional up regulation of its gene. Activation of
Rac1, in addition to triggering intracellular production of $\text{O}_2^-$, has also been shown to regulate actin polymerization and Jun kinase (JNK) activation (Campbell SL et al, 1998). In order to assess that Rac1-mediated induction of NHE-1 gene promoter activity was a function of an increase in intracellular $\text{O}_2^-$, loss-of-function mutants of Rac1 containing specific amino acid substitutions in the activated RacV12 background (Pervaiz S et al, 2001) were used. The RacV12L37 mutant activates JNK but is defective in inducing actin polymerization, whereas the RacV12H40 mutant induces actin polymerization but is defective in JNK activation. However, both of these mutations still retain the ability to generate intracellular $\text{O}_2^-$ (Joneson T and Bar-Sagi D, 1998). In contrast, the RacV12H103 and RacV12K166 are deficient in $\text{O}_2^-$ production (Toporik A et al, 1998). Using Rac loss-of-function mutants it became clear that JNK activation and actin polymerization function of Rac was not involved in the survival advantage, and it was the $\text{O}_2^-$ producing function of Rac. NIH3T3 cells over expressing pIRES-RacV12, pIRES-RacV12H40 or pIRES-RacV12L37 were more resistant to cell death as compared to cells transfected with pIRES control vector or the Rac mutants deficient in $\text{O}_2^-$ production (pIRES-RacV12H103A and pIRES-RacV12K166E). In the light of our findings, it is plausible that in addition to a phosphorylation-dependent activation of NHE-1, $\text{O}_2^-$-dependent transcription of the NHE-1 gene may be a critical regulator of cell survival. Transient transfection with dominant negative form of Rac1, RacN17, showed that serum-induced activation of NHE-1 might involve Rac1. These results could have important relevance in tumor cells whereby an activated Rac1 could lead to increased $\text{O}_2^-$ levels and up regulation of NHE1 protein expression.
IV.11. $\text{H}_2\text{O}_2$ inhibits NHE-1 promoter activity and leads to increased susceptibility to cell death

Intracellular concentration of $\text{O}_2^{-}$ is tightly regulated in mammalian cells by the superoxide dismutase (SOD) enzymes, i.e. the Cu/Zn superoxide dismutase (Cu/ZnSOD) in the cytoplasm and MnSOD in the mitochondria, which dismutate $\text{O}_2^{-}$ into $\text{H}_2\text{O}_2$ that is subsequently transformed into $\text{H}_2\text{O}$ and $\text{O}_2$ by catalase or GSH peroxidases. As it is known that redox regulated transcription factors such as NFkB and AP-1 could be activated by $\text{H}_2\text{O}_2$ (Baeuerle PA and Baltimore D, 1996; Bowie A and O'Neill LA, 2000), we asked whether the increase in NHE-1 promoter activity in our system was indeed due to $\text{O}_2^{-}$ or a function of its dismutated product, $\text{H}_2\text{O}_2$.

Our results show clearly that non toxic concentration of $\text{H}_2\text{O}_2$ was unable to activate the NHE-1 promoter. Instead, incubation of NIH3T3 1A8 cells with increasing concentration of $\text{H}_2\text{O}_2$ resulted in inhibition of NHE-1 promoter activity. These results indicate that $\text{O}_2^{-}$, and not $\text{H}_2\text{O}_2$ is the reactive oxygen species responsible for the regulation of NHE-1 gene expression. Moreover, the use of two different reporter gene systems rules out the possibility that the observed inhibitory effect on NHE-1 promoter activity could have been attributed to the inhibitory effect of $\text{H}_2\text{O}_2$ on the reporter gene (luciferase or CAT) itself. However, the inhibition of the NHE-1 promoter activity by $\text{H}_2\text{O}_2$ could either be due to direct inhibition of $\text{H}_2\text{O}_2$ on NHE-1 specific transcription factors or via an indirect effect on the level of $\text{O}_2^{-}$. In this regard, it has been previously shown that production of $\text{H}_2\text{O}_2$ could induce a decrease in intracellular $\text{O}_2^{-}$ level by over-activation of the nuclear protein poly (ADP)-ribose polymerase (Clement MV et al, 1998). Hence, inhibition of the NHE-1 promoter activity could be a consequence of a decrease in intracellular $\text{O}_2^{-}$ concentration. In addition, results showing that inhibition of Cu/Zn SOD with DDC resulted in a
significant activation of the promoter also favor $\text{O}_2^-$ rather than $\text{H}_2\text{O}_2$ in the activation of the NHE-1 gene transcription. It should be pointed out that a previous report had demonstrated that $\text{H}_2\text{O}_2$ was an inducer of NHE-1 gene transcription in rat cardiomyocytes (Gan XT et al, 1999). This observed conflict could be due to involvement of different redox-regulated transcription factors in myocytes compared to cells used in this study.

Moreover, similar to the result obtained by silencing NHE-1, pre-incubation of NIH3T3 cells with $\text{H}_2\text{O}_2$ sensitized NIH3T3 cells to etoposide-induced cell death. Taken together, these data provide strong evidence to support that the expression of NHE-1 is redox regulated, with opposing effects of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ on its promoter activity and protein expression.

IV.12. Regulation of intracellular pH as one of the mechanisms of NHE-1-mediated cell survival

Activation of NHE-1 activity has been mainly attributed to direct activation of kinases leading to NHE-1 phosphorylation. In the light of present study, it wouldn’t be wrong to speculate a scenario in tumor cells where a persistent increase in intracellular $\text{O}_2^-$ would lead to a higher basal expression of NHE-1 protein. Hence, in tumor cells this persistent increase could lead to a more alkaline “set point” of the NHE-1 exchanger due to increased transcription of the NHE-1 gene. This hypothesis is supported by the results in M14 and U87 cells. Upon silencing of NHE-1 gene it was observed that steady state pH$i$ dropped significantly. This is in contrary to experiments where NIH3T3 cells were used and little or no difference was observed between control and NHE-1 silenced cells. In addition, when NHE-1 protein was over-expressed in NIH3T3 cells, still no significant change was seen in pH$i$. Hence it can be concluded
here that over-expression of a quiescent H⁺ pump is not expected to significantly alter steady state pH in normal cells. On the other hand, tumor cells seem to behave in a different manner. Expression of an activated form of Rac1 in M14 tumor cells seems to be responsible for the increase in NHE-1 protein level leading to a significant alkanization of the cytosol, while silencing NHE-1 expression had the opposite effect (acidification). Hence, a sustained increase in intracellular O₂⁻ by activation of the small GTPase Rac could lead to a more alkaline “set point” of the NHE-1 exchanger due to increased transcription of the NHE-1 gene. Increase in the “set point” of the antiporter was described previously during HL60 cells differentiation (Rao GN et al, 1993); differentiation of HL60 cells was accompanied by an increase in the cells’ basal pH from 7.15 to 7.26. However, this increase in pH could not be attributed to phosphorylation of the exchanger. In addition, constitutive expression of RacV12 in M14 cells also correlated with resistance to apoptotic cell death (Pervaiz S et al, 2001). Hence, it is tempting to speculate that O₂⁻-mediated activation of the NHE-1 promoter activity could couple intracellular alkanization with cell survival induced by activation of the small GTPase Rac1. Altogether, these findings could have tremendous implications in enhancing our understanding of the role of NHE-1 in tumorigenesis. It must be pointed out at this stage that although in tumor cells (U87 and M14) a better pH difference was observed when NHE-1 protein expression was altered; it merits further investigation in order to elucidate the exact role of pH in tumorigenesis. Moreover recent evidence strongly suggests that, in addition to regulating pH NHE-1 could be a critical regulator of cell sensitivity to apoptotic stimuli (Wu KL et al, 2003). Therefore, we hypothesized that the functional consequence of the redox regulation of NHE-1 protein expression could be linked to the regulation of cell sensitivity to death triggers. Regulation of pH might be one of
the mechanisms downstream of NHE-1 that contributes to cell death resistance. Other possible mechanisms downstream of NHE-1 could be activation of survival pathways including PI3k/Akt pathway (Wu KL et al, 2004). As a lot of functions have been recently attributed to NHE-1 protein, it wouldn’t be wrong to speculate that pH-modulating function of NHE-1 may or may not be directly involved in its ability to induce survival in tumor cells.

IV.13. Region of NHE-1 promoter involved in O$_2^-$-mediated activation

NHE-1 gene is a large gene comprising of approximately 70 kilobases and can be regulated by a variety of stimuli. There is strong homology (>90%) between human and mouse NHE-1. As it became clear that O$_2^-$ acts as a signal for NHE-1 expression, and this increased expression of NHE-1 protein might play an important role in tumor cells, our next question was that where does O$_2^-$ act on the NHE-1 promoter and what are the candidate transcription factors. We used L6 rat muscle cells stably transfected with truncated versions of NHE-1 promoter. As was demonstrated by Besson et al. that 1.1 kb is the minimum length of promoter needed for maximum activity (Besson P et al, 1998), we used L6 cells stably transfected either with 1.1 kb, 0.9 kb, 0.5 kb, 0.18 kb or AP2+ constructs. The AP2+ construct is 147 base pairs in length and it retains the AP2 binding site. Using either paraquat as a trigger for intracellular O$_2^-$ or RacV12 expression, we demonstrate that O$_2^-$ seems to act somewhere between the 0.5 to 0.2 kb proximal fragment of mouse NHE-1 promoter. The region between 0.5 kb to 0.2 kb of mouse promoter correlates to an area of human NHE-1 promoter designated as area D. Evidence indicates that a number of regions of NHE-1 promoter contribute to the basal expression of the gene (Kolyada AY et al, 1998). In addition,
DNA footprinting experiments have revealed that different regions bind proteins of nuclear extract (Dyck JRB and Fliegel L, 1995).

AP-1 was the first transcription factor identified to induce NHE-1 gene transcription. AP-2 transcription factor has been shown to be a key player (Dyck JRB and Fliegel L, 1995; Rieder CV and Fliegel L, 2003). The chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are orphan receptors involved in regulation of embryonic development and neuronal cell fate determination. Fernandez-Rachubinski et al. have shown that a nuclear hormone responsive element located at -841/-800 nt of the mouse NHE-1 promoter binds COUP-TF with enhancer activity (Fernandez-Rachubinski F et al., 2001). Thyroid hormone and thyroid hormone receptor alpha 1 (TRα1) has also been shown to regulate NHE-1 gene expression (Li X et al, 2002). Thus, it becomes clear that many transcription factors can influence NHE-1 promoter. Keeping in view the importance of NHE-1 in cell growth and differentiation, there might be many more elements affecting its expression. Much needs to be done to identify the candidate transcription factor(s) downstream of \( \text{O}_2^- \) production in the cell. Identification and characterization of this element will provide us with a better understanding of redox-mediated regulation of NHE-1 gene.

IV.14. Conclusions

In summary, the results presented here provide strong evidence to implicate intracellular redox status in the regulation of NHE-1 promoter activity and gene expression. This report demonstrates that NHE-1 is a redox-regulated gene, and the effect of \( \text{O}_2^- \) on NHE-1 gene transcription was demonstrated (a) by inhibiting \( \text{O}_2^- \) production using DPI, a widely used inhibitor of \( \text{O}_2^- \)-producing oxidase, (b) by increasing the level of intracellular \( \text{O}_2^- \) using DDC, an inhibitor of the Cu/Zn SOD.
Activated Rac1
DDC
Paraquat

Serum withdrawal
DPI
Tiron

Increased O$_2^-$ production

H$_2$O$_2$

Increased NHE1 transcription

NHE1 silencing
NHE1 transfection

Increased NHE1 protein/activity

? pH$i$

Inhibition of Apoptosis

Figure 38
and (c) by showing that activation of NHE-1 promoter activity by the small GTPase Rac1, a widely accepted essential component for oxidase-mediated production of intracellular $\text{O}_2^\cdot$. Moreover, we show that level of NHE-1 protein expression can influence cell sensitivity to death triggers. An increase in NHE-1 protein expression downstream of $\text{O}_2^\cdot$ production was able to bestow a survival advantage to the cell in response to apoptotic triggers, and vice versa. Interestingly H$_2$O$_2$ caused repression of the NHE-1 promoter and reversal of resistant phenotype. These observations are summarized in Figure 38. These findings could provide a mechanism for the regulation of cell survival by ROS, particularly in tumor cells, and may be important in enhancing our understanding of the relationship between cell redox status and tumorigenesis. However, apart from the possible oxidation mediated inhibition of key enzymes involved in tumor cells’ death, the exact mechanism involved in $\text{O}_2^\cdot$-mediated cell survival is not yet well understood. Data presented here support that NHE-1 could be one such target. Indeed, in absence of NHE-1 gene expression DDC was unable to inhibit staurosporine-induced cell death in NIH3T3 cells despite inducing an increase in intracellular level of $\text{O}_2^\cdot$.

NHE-1-mediated inhibition of cell death has mostly been attributed to its ability to extrude H$^+$ upon death triggers, and thereby create an intracellular environment non-permissive for death execution. Recent evidence has pointed out towards many other important functions of NHE-1 protein in addition to its primary function of pH modulation. Two recent reports have demonstrated that activation of NHE-1 induced pro-survival kinase PKB/AKT in human renal proximal tubule epithelial cells (Wu KL et al, 2004) and ERK activity in rat aortic vascular smooth muscle cells (Mukhin et al., 2004). In the light of these findings and our data presented here, we propose that redox regulation of NHE-1 expression could indirectly influence activation of
two well-known survival pathways, i.e. the PI3K/AKT and ERK. These extremely interesting findings point towards a multi-faceted role NHE-1 might play in a cell, rather then simply being a pH-regulator. Thus, it wouldn’t be wrong to speculate that the NHE-1 mediated cell survival could or could not be dependent upon its primary function of pH modulation. It has also been shown that paclitaxel induces apoptosis in breast cancer cells via protein kinase A and p38 MAPK-dependant repression of NHE-1 (Reshkin SJ et al, 2003). In light of these recent findings, the present report could have tremendous implications in our understanding of redox regulated sensitivity of cells to death triggers and may uncover the molecular mechanism involved in redox-mediated survival in normal and tumor cells.

IV.15. Prospective studies

In the light of current findings, it is probable to plan for further studies in this important area of cancer research. It would be interesting to find out the region of NHE-1 promoter responsive to the superoxide signal, as it can help to discover the transcription factor(s) involved. Insight into the mechanism of increased cell survival upon increased expression of NHE-1 protein could shed light on the vital role this exchanger might play in tumor cell resistance to apoptotic signals. The cell survival ability of NHE-1 could be independent of its pH modulation function, and this area is currently a focus of research in many laboratories.
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AWARDS AND SCHOLARSHIPS

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(awarded at the 6th NUS-NUH Annual Scientific Meeting)

August 2002

National University of Singapore Research Scholarship

Jun 2000 - Jun 2004