

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inflammatory Cytokines Induce Human Bronchial Smooth Muscle Cell Proliferation via
an NCX-1 Dependent Mechanism

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
DG	diacylglycerol
ER	endoplasmic reticulum
DMSO	dimethyl sulfoxide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
HASMC	human airway smooth muscle cells
HBSMC	human bronchial smooth muscle cells
IL-13	interleukin-13
IL-33	interleukin-33
IP₃	inositol triphosphate
MLC	myosin light chain
MLCK	myosin light chain kinase
NCX1	Na ⁺ /Ca ²⁺ exchanger 1
NCKX	Na ⁺ /Ca ²⁺ /K ⁺ exchanger
Ova	ovalbumin
PIP₂	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
ROC	Receptor operated channel
RT-PCR	Reverse transcriptase polymerase chain reaction
SOC	store operated channel
SR	sarcoplasmic reticulum
T_H2	T-helper cell type 2
TNF	tumor necrosis factor
TRP	transient receptor potential
VGCC	voltage gated calcium channel

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ABSTRACT OF THE THESIS

Inflammatory Cytokines Induce Human Bronchial Smooth Muscle Cell Growth via an NCX-1 Dependent Mechanism

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Master of Science in Biology

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Airway smooth muscle hyperplasia is a characteristic of airway remodeling in asthma and this is thought to be, at least in part, cytokine mediated. Because cytosolic free calcium ($[Ca^{2+}]_{cyt}$) plays an important role in smooth muscle proliferation, we chose to examine the role of $[Ca^{2+}]_{cyt}$, focusing on the expression of the Na^+/Ca^{2+} exchanger 1 (NCX1) protein and its link to human airway smooth muscle proliferation.

In vitro studies were done to examine the function and expression of NCX1 protein in human bronchial smooth muscle cells (HBSMC). Cells were grown in the presence/absence of the inflammatory cytokines $TNF\alpha$, IL-13, and IL-33 and assessed for proliferation using a colorimetric assay. Proliferation was induced in the presence of

inflammatory cytokines and blocked in the presence of SN-6, a selective NCX1 inhibitor. Immunoblotting, immunocytochemistry, and quantitative PCR revealed that inflammatory cytokines upregulate NCX1 protein and mRNA expression. *In vivo* studies were done in mice using an ovalbumin model of asthma. NCX1 expression in asthmatic mice airway was compared with control mice. Immunoblotting revealed a substantial increase in NCX1 protein expression in asthmatic mice.

We have demonstrated that NCX1 is expressed in HBSMC and that this expression is increased by cytokines associated with asthma. Moreover, cells proliferate with these cytokines, which is blocked by NCX1 inhibition. NCX1 is also expressed and upregulated in asthmatic mice airway. These data suggest that NCX1 may play an important role in airway remodeling associated with asthma.

Introduction

Asthma Pathophysiology

Asthma is a disease of the airway, characterized by inflammation, hyperresponsiveness, and remodeling (Barrios, et al. 2006). It affects over twenty million people in the United States (6% of the population), and is growing in prevalence. Airway hyperresponsiveness is the occurrence of excessive bronchoconstriction in response to a given dose of agonist. Airway remodeling consists of substantial thickening of both the airway subepithelial and smooth muscle layers (Leguillette, et al. 2008). This is a result of increased airway smooth muscle proliferation (hyperplasia) as well as increased smooth muscle size (hypertrophy) (Bentley, et al. 2008). Airway obstruction is triggered by allergic immune response. T helper cell type 2 (T_H2 Cells) secretes cytokines including IL-4, IL-5, IL-9, and IL-13, which contribute to allergic inflammation (Barrios, et al. 2006). TNF α and IL-33 are also inflammatory cytokine present during an allergic immune response. TNF α is released by macrophages. IL-33 is released by macrophages, monocytes, endothelial cells, and epithelial cells. IL-33 induces expression of T_H2 cytokines, and is upregulated by TNF α (Prefontaine, et al. 2009) in human airway smooth muscle.

Calcium Signaling in Airway Smooth Muscle

Free cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) in smooth muscle controls a variety of functions including contraction, secretion, transcription, cell division, and cell death (Berridge, 2006; Orrenius, et al. 2003). Increased $[Ca^{2+}]_{cyt}$ in human airway smooth muscle (HASM) triggers contraction, cell proliferation and migration (Sweeney, et al.

2002). Contraction of ASM occurs through the interaction of the proteins actin and myosin. When $[Ca^{2+}]_{cyt}$ increases, free Ca^{2+} binds to calmodulin. The Ca^{2+} /calmodulin complex activates myosin light chain kinase (MLCK), which then phosphorylates the myosin light chain (MLC). Phosphorylated myosin can then bind actin. Myosin ATPase causes energy to be released from ATP, resulting in smooth muscle contraction through the cycling of myosin cross-bridges with actin (Webb, 2003).

$[Ca^{2+}]_{cyt}$ increases as a result of Ca^{2+} release from intracellular stores (sarcoplasmic reticulum), as well as from the extracellular space via membrane Ca^{2+} channels. When agonists such as acetylcholine, norepinephrine, angiotensin II, and endothelin bind to G-protein coupled receptors (GPCR's), a signal cascade occurs beginning with the activation of phospholipase C (PLC). PLC cleaves the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol triphosphate (IP_3) and diacylglycerol (DG). IP_3 binds to receptors on the sarcoplasmic reticulum (SR), resulting in the release of Ca^{2+} into the cytoplasm. DG and free Ca^{2+} activate protein kinase C (PKC) which phosphorylates calcium channels on the cell membrane, resulting in Ca^{2+} influx. Relaxation of smooth muscle is achieved by pumping Ca^{2+} back into the sarcoplasmic reticulum stores or out across the plasma membrane. In addition to controlling smooth muscle cell contraction, an increase in $[Ca^{2+}]_{cyt}$ in other airway cells such as airway epithelial cells would stimulate the release of inflammatory mediators. $[Ca^{2+}]_{cyt}$ in airway smooth muscle also controls mitochondrial biogenesis, which in turn controls proliferation (Trian, et al. 2007). Due to all the above functions of $[Ca^{2+}]_{cyt}$, it can be implied that abnormal $[Ca^{2+}]_{cyt}$ homeostasis

in airway cells may contribute to airway hyperresponsiveness, remodeling, and inflammation (Sweeney, et al. 2002).

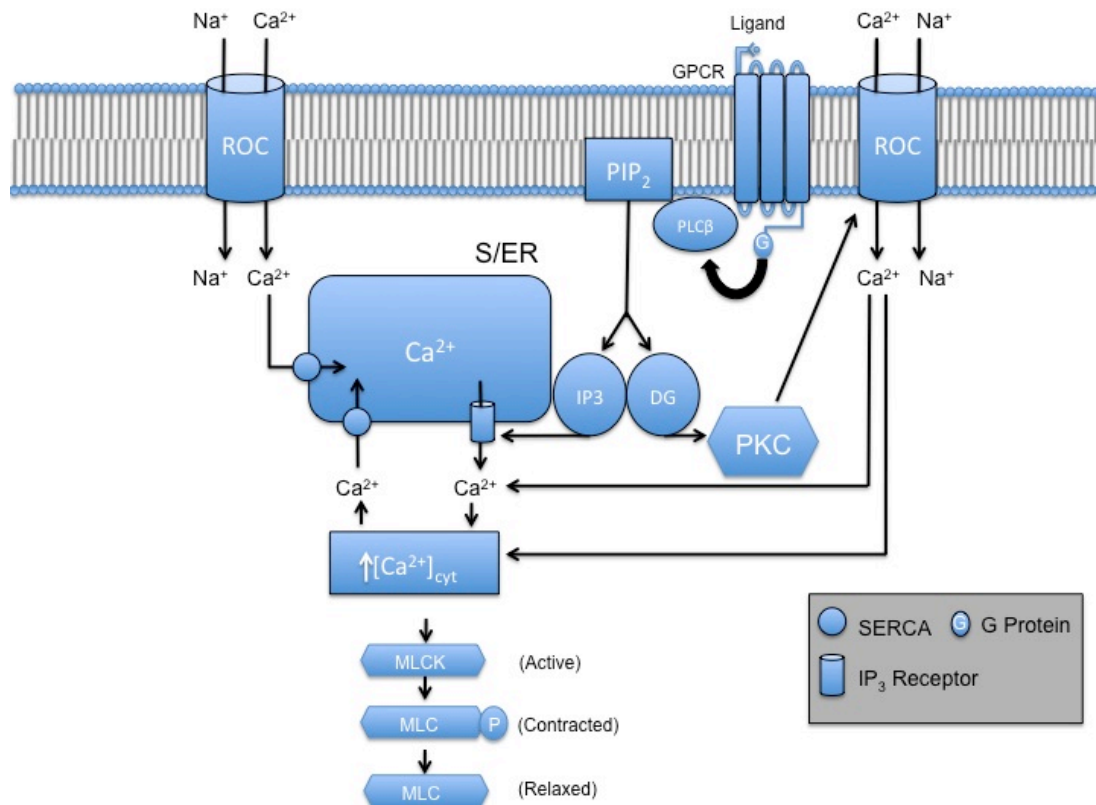


Figure 1. Regulation of smooth muscle contraction. Various agonists (neurotransmitters, hormones, etc.) bind to specific receptors to activate contraction in smooth muscle. Subsequent to this binding, the prototypical response of the cell is to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces two potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃). IP₃ binds to specific receptors on the sarcoplasmic reticulum, causing release of activator calcium (Ca²⁺). DG along with Ca²⁺ activates PKC, which phosphorylates specific target proteins. In most smooth muscles, PKC has contraction-promoting effects such as phosphorylation of Ca²⁺ channels or other proteins that regulate cross-bridge cycling. Activator Ca²⁺ binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase). This kinase phosphorylates the light chain of myosin, and in conjunction with actin, cross-bridge cycling occurs; initiating shortening of the smooth muscle cell (Webb, 2003).

Plasma Membrane Ca²⁺ Channels in Airway Smooth Muscle

There are at least three classes of plasma membrane Ca²⁺ channels involved in [Ca²⁺]_{cyt} homeostasis. Voltage-dependent Ca²⁺ channels (VGCC), receptor-operated Ca²⁺ channels (ROC), and store-operated Ca²⁺ channels (SOC) (Kotlikoff, et al. 1999). Membrane potential controls Ca²⁺ influx via voltage-dependent Ca²⁺ channels (Fleischmann, et al. 1994). Out of the three previously mentioned classes of plasma membrane Ca²⁺ channels, the VGCC's contribute the least to [Ca²⁺]_{cyt} (Barnes, et al. 1998). ROC's and SOC's, in addition to SR calcium channels, play more important roles in controlling [Ca²⁺]_{cyt}. ROC's operate via the previously discussed GPCR pathway. ROC's are activated by PKC's following GPCR activation by agonists. They increase [Ca²⁺]_{cyt} in a biphasic manner consisting of an initial transient increase in [Ca²⁺]_{cyt}, followed by a sustained [Ca²⁺]_{cyt} increase. Sustained rise in [Ca²⁺]_{cyt} depends upon the continued binding of the agonist and also depends on extracellular Ca²⁺ (Murray, et al. 1991).

SOC's are activated by depletion of the SR Ca²⁺. Depletion of the SR Ca²⁺ triggers capacitative Ca²⁺ entry (CCE), a mechanism involved in maintaining sustained Ca²⁺ influx and refilling Ca²⁺ in the SR. Decreased SOC expression attenuated HASMC proliferation and contraction, implying that SOC's play important roles in controlling bronchial constriction and HBSMC proliferation (Sweeney, et al. 2002). The molecular identities of SOC and ROC have still not been completely revealed, but work in this area has focused on transient receptor potential (TRP) channels, based on observations that TRP expression leads to Ca²⁺ channels triggered by store depletion and receptor activation (Ma, et al. 2000; Venkatachalam, et al. 2002).

TRP Channels in Airway Smooth Muscle

TRP channels contains several members (TRPC1, TRPC3, and TRPC6) which may function as SOC's or ROC's in HASMC's (Sweeney, et al. 2002; White, et al. 2006). Expression of TRPC genes in mammalian cells results in the formation of Ca^{2+} -permeable channels that are activated by SR Ca^{2+} depletion which allow CCE. Despite that TRPC channels in HASMC's have been implicated in HASMC contraction, proliferation, and remodeling (Sweeney, et al. 2002), their direct involvement in asthma remains elusive. Human airway myocyte activation through TRPC3 has recently been reported to be increased by proinflammatory cytokine, $\text{TNF}\alpha$, suggesting the upregulation of TRPC3, and also suggesting involvement in asthma (White, et al. 2006). TRP channels are generally nonselective cation channels that allow both Ca^{2+} and Na^+ to diffuse into the cytoplasm, resulting in an increase in both cytoplasmic Ca^{2+} and Na^+ concentrations (Zhang, et al. 2005). Since TRPC-encoded SOC and/or ROC are found to be functionally coupled to $\text{Na}^+/\text{Ca}^{2+}$ exchangers, their upregulation not only emphasizes the importance of $[\text{Ca}^{2+}]_{\text{cyt}}$ in HASMC's, but also implies that $\text{Na}^+/\text{Ca}^{2+}$ exchangers may play a role in asthma (White, et al. 2006).

$\text{Na}^+/\text{Ca}^{2+}$ exchangers

The $\text{Na}^+/\text{Ca}^{2+}$ exchangers are ion transport proteins. They are integral plasma membrane proteins that exchange Na^+ for Ca^{2+} (or plus K^+). Two families of $\text{Na}^+/\text{Ca}^{2+}$ exchangers have been described in mammalian cells. NCX1-3 depends only on Na^+ flux. NCKX1-6 depends on potassium in addition to sodium. NCX1 is expressed in many cells types such as cardiac muscle, epithelia, and smooth muscle (Blaustein, et al. 1999).

$\text{Na}^+/\text{Ca}^{2+}$ exchangers are transmembrane proteins. It contains an intracellular loop, which may be involved in modulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger function through intracellular kinases, as well as ions. Calcium binds in the middle of the intracellular loop region of the protein. $\text{Na}^+/\text{Ca}^{2+}$ exchangers contain six transmembrane regions at the carboxy terminal end, followed by a large intracellular loop region, followed by five transmembrane regions and a signal sequence at the amino terminus. The amino terminus is located in the extracellular space. The intracellular loop contains a Na^+ inactivation region, a Ca^{2+} binding region, and an alternatively spliced region (Blaustein, et al. 1999).

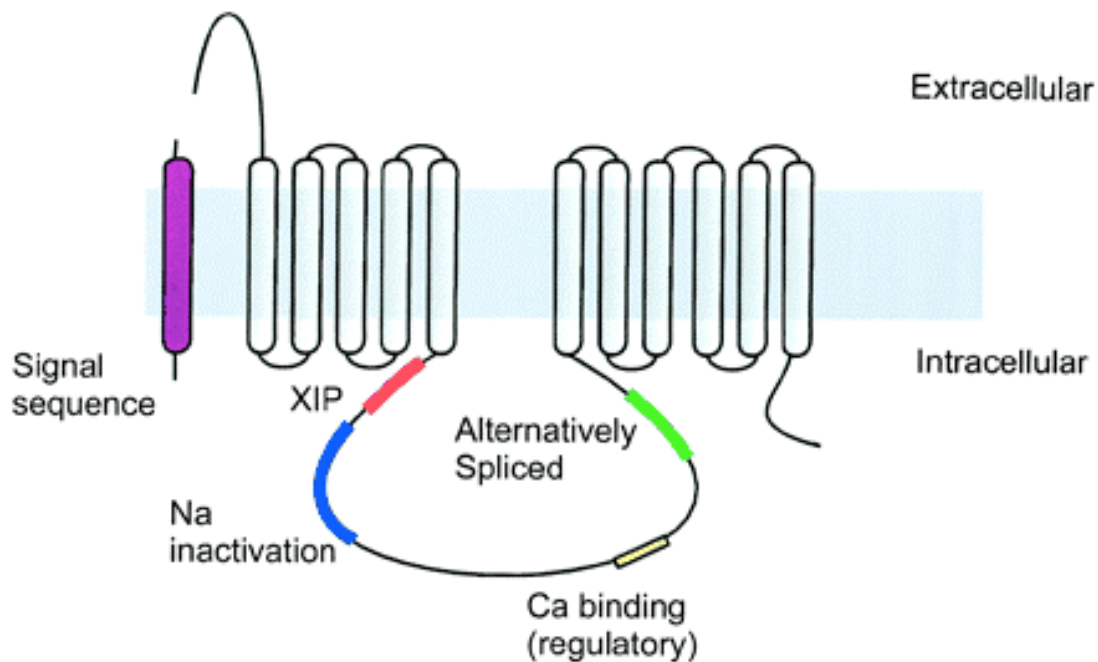


Figure 2. Model of $\text{Na}^+/\text{Ca}^{2+}$ exchanger protein.

Transmembrane-spanning regions are identified (light blue = PM), as are XIP region (red), Na^+ -inactivation region (green), Ca^{2+} -binding regions (yellow), and alternatively spliced region (dark blue) (Blaustein, 1999).

$\text{Na}^+/\text{Ca}^{2+}$ exchangers operate in either forward mode (calcium exit) or reverse mode (calcium entry), depending on sodium, calcium, and potassium gradients, as well as the membrane potential. The reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchangers has been suggested to contribute to Ca^{2+} homeostasis. In most cells, three Na^+ are exchanged for one Ca^{2+} . In cardiac myocytes as well as other cell types, NCX1 maintains a low $[\text{Ca}^{2+}]_{\text{cyt}}$. When $[\text{Na}^+]_{\text{cyt}}$ increases, $[\text{Ca}^{2+}]_{\text{cyt}}$ increases as a result of $\text{Na}^+/\text{Ca}^{2+}$ exchange via the reverse mode of NCX. For a constant extracellular $[\text{Ca}^{2+}]$ (1.8-2mM) and $[\text{Na}^+]$ (~140 mM), $[\text{Ca}^{2+}]_{\text{cyt}}$ is a cubic function of $[\text{Na}^+]_{\text{cyt}}$ and an exponential function of membrane potential. Therefore, a small change in $[\text{Na}^+]_{\text{cyt}}$ or membrane potential can cause large changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Blaustein, et al. 1999).

Blaustein and van Breeman have recently proposed a working model for Ca^{2+} entry via the reverse mode of NCX under physiological conditions. Openings of TRP-encoded ROC's and SOC's via the DG-PKC pathway (activates ROC's) and the PLC-IP3 pathway (activates SOC's through store depletion) result mainly in Na^+ influx into the cell, causing it to depolarize. The depolarization and increased Na^+ drive NCX into its reverse mode of operation, increasing $[\text{Ca}^{2+}]_{\text{cyt}}$. The reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchangers plays a significant role in vascular contraction, cell proliferation, and pulmonary arterial hypertension (Dong, et al. 2006; Zhang, et al. 2005).

Role of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in the airway

Although $\text{Na}^+/\text{Ca}^{2+}$ exchangers have been extensively studied in cardiovascular and nervous systems, its role in the respiratory system remains elusive. So far, studies of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in the airway have been contradictory. Some studies have shown that $\text{Na}^+/\text{Ca}^{2+}$ exchangers contribute to SR Ca^{2+} refilling (Dai, et al. 2006; Hirota, et al.

2006), while others studies claim that $\text{Na}^+/\text{Ca}^{2+}$ exchangers do not play a significant role in store refilling and smooth muscle contraction (Knox, et al. 1994; Janssen, et al. 1997). $\text{Na}^+/\text{Ca}^{2+}$ exchangers have been shown to be functionally expressed in mast cells and contribute to antigen-stimulated degranulation and secretion of inflammatory mediators from mast cells (Rumpel, et al. 1995). However, the role of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in mast cells in asthma is unknown. The location and physiology of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in the respiratory system have not been studied. Therefore, preliminary studies of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in murine and human airway were conducted.

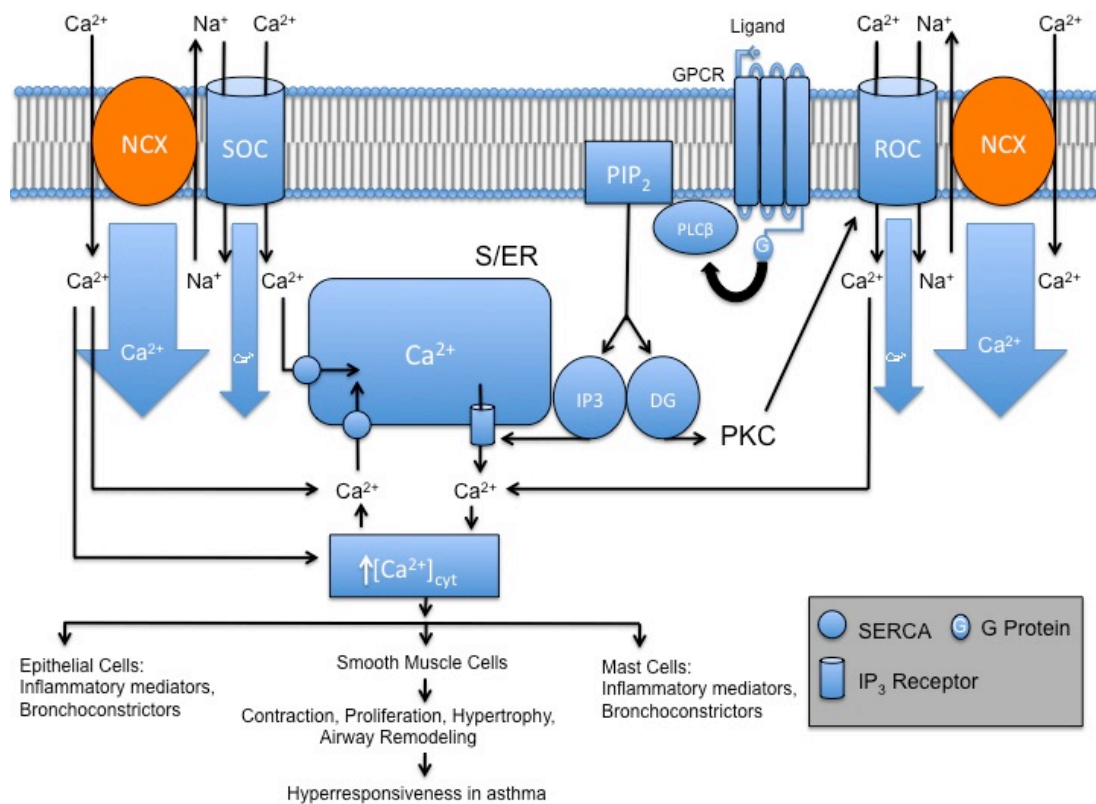


Figure 3. A proposed model for the involvement of NCX in the pathogenesis of asthma. NCX: $\text{Na}^+/\text{Ca}^{2+}$ exchanger, SOC: store-operated channels, S/ER: Sarco(endo)plasmic reticulum, PLC: protein kinase C, DG: diacylglycerol. Blue arrows are representative of relative contributions to $[\text{Ca}^{2+}]_{\text{cyt}}$.

Preliminary Studies from Dong and Bigby Labs

To determine which $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoforms were expressed in murine airway smooth muscle, northern blotting was done on rat tissues. The northern blots revealed that mRNA of NCX1, NCKX3 and NCKX4 are expressed in smooth muscle, and also that NCKX1 and NCKX2 are not expressed significantly in airway smooth muscle (Figure 4).

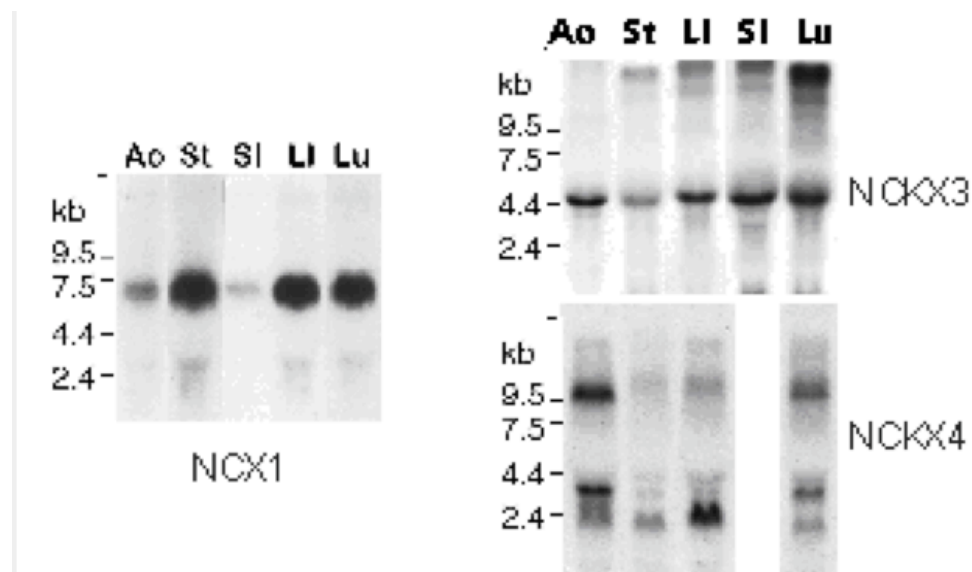


Figure 4. Expression of mRNA of NCX1, NCKX3 and NCKX4 in smooth muscle. mRNA in smooth muscle tissues was analyzed by Northern blotting. Ao: aorta, St: stomach, SI: small intestine, LI: large intestine and Lu: lung with bronchia.

Western blots were conducted using R3F1, an anti-NCX1 monoclonal antibody. R3F1 detected two proteins with molecular masses of 120 and 70 kDa in the murine trachea and lung, corresponding to previous reports of native NCX1 protein (Figure 5A). In order to see whether or not NCX1 activity contributed to tracheal function, tracheal rings isolated from mice were mounted in organ baths, and their contractility was

determined with a transducer and recorded with a polygraph. Carbachol (CCh), a muscarinic receptor agonist, induced two similar tracheal contractions in normal physiological salt solution (Figure 5B). When external Na^+ was removed, carbachol induced contraction was significantly attenuated (Figure 5B). KB-R7943, a specific inhibitor for the reverse mode of NCX, further inhibited the carbachol-induced tracheal contraction. These studies revealed that expression of NCX1 proteins and measurement of tracheal contractility are consistent with functional expression of the reverse mode of NCX1 in murine airway (Dai, et al. 2006; Hirota, et al. 2006).

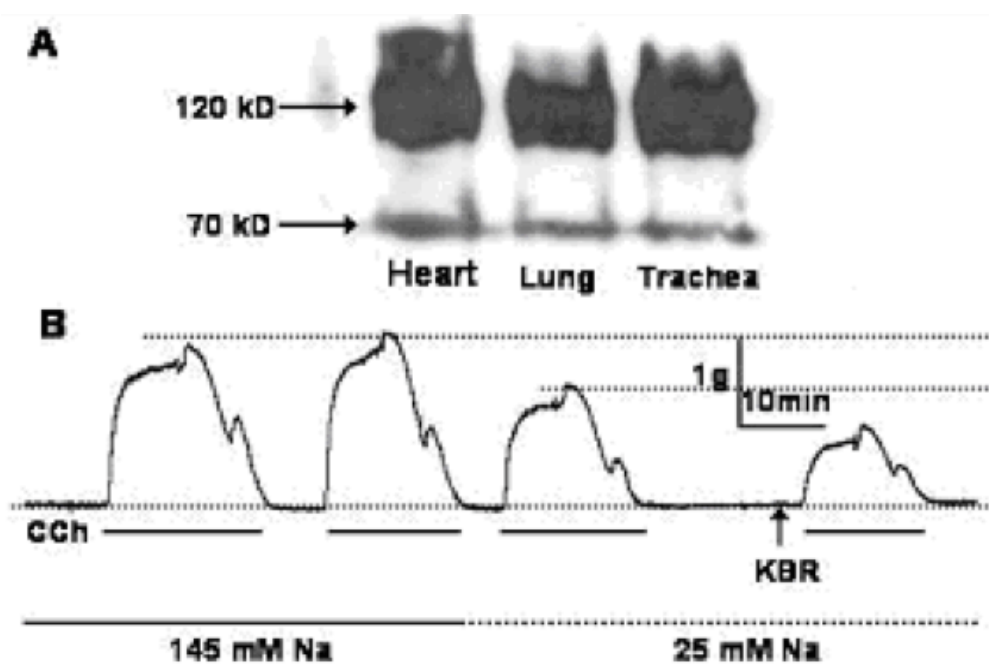


Figure 5. The expression (A) and function (B) of NCX1 protein in the murine respiratory system. A) Western blots were conducted using R3F1, an anti-NCX1 monoclonal antibody. R3F1 detected two proteins with molecular masses of 120 and 70 kDa in the murine trachea and lung, corresponding to previous reports of native NCX1 protein. B) Carbachol induced tracheal contraction that was attenuated with Na^+ removal. KB-R7943, a specific inhibitor for the reverse mode of NCX, further inhibited tracheal contraction.

In order to functionally identify the reverse mode of NCX1 in single cells from primary cultures of mouse airway smooth muscle cells, $[\text{Ca}^{2+}]_{\text{cyt}}$ was measured in single

cells from primary cultures of mouse tracheal smooth muscle cells. CCh induced a significant increase in $[Ca^{2+}]_{cyt}$ in the cells. Cells were then pretreated with KB-R7943 and then perfused with CCh. KB-R7943 markedly attenuated CCh-induced increase in $[Ca^{2+}]_{cyt}$ (Figure 6). This demonstrated that the reverse mode of NCX1 contributed to the CCh induced calcium influx. Another experiment was conducted where extracellular Na^+ was removed in order to activate the reverse mode of NCX1. This induced a rapid increase in $[Ca^{2+}]_{cyt}$, which was attenuated using KB-R7943 (Figure 7). These data suggest that the reverse mode of NCX1 plays an important role in controlling $[Ca^{2+}]_{cyt}$ homeostasis in single ASMC.

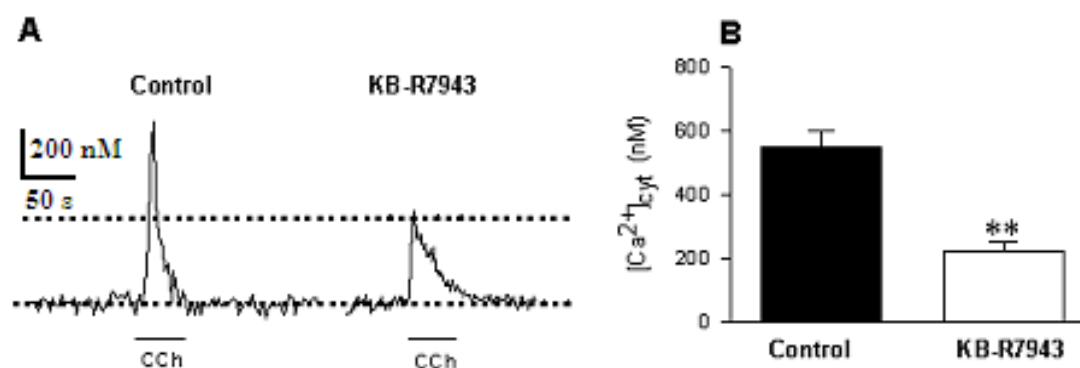


Figure 6. The reverse mode of NCX contributes to CCh-induced increase in $[Ca^{2+}]_{cyt}$ in single cell measurements from primary cultures of mouse ASMC. A) Representative records showing the time course of CCh-induced $[Ca^{2+}]_{cyt}$ changes in ASMC (left panel) and inhibition by 10 μ M KB-R7943 (right panel). B) Summarized data showing amplitude of $[Ca^{2+}]_{cyt}$ increases in control cells and pretreated with KB-R7943. ** $P < 0.01$, $n = 20$ cells for each group.

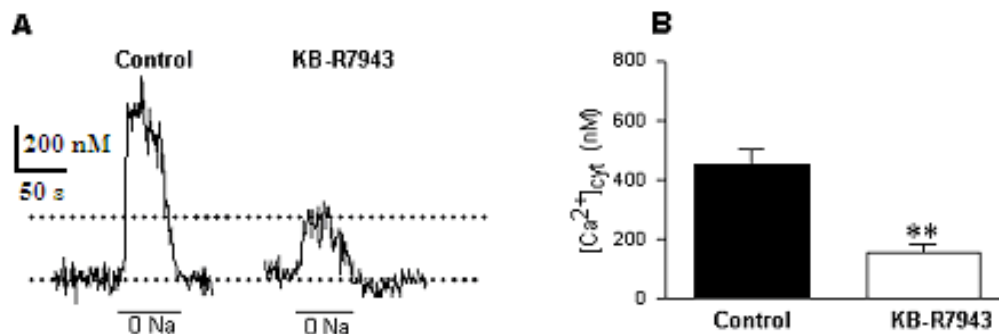


Figure 7. The reverse mode of NCX contributes to removal of external Na-induced increase in $[Ca^{2+}]_{cyt}$ in single cell measurements of primary cultures of mouse ASMC. *A*) Representative records showing the time course of 0 Na-induced $[Ca^{2+}]_{cyt}$ changes in ASMC (left panel) and inhibition by 10 μ M KB-R7943 (right panel). *B*) Summarized data showing amplitude of $[Ca^{2+}]_{cyt}$ increases in control cells and pretreated with KB-R7943. ** $P < 0.01$, $n = 30$ cells for each group.

Expression levels of NCX1 protein were compared in lungs from normal mice and lungs from asthmatic mice. NCX1 proteins were found to substantially increased in bronchial smooth cells from asthmatic mice (Figure 8). Since the protein expression of NCX1 is upregulated in bronchial cells of asthmatic mouse model, it was hypothesized that upregulation of NCX1 proteins contributes to the enhanced Ca^{2+} entry in respiratory cells and evokes asthma. Therefore, KB-R7943, a specific inhibitor of the reverse mode of NCX was given to mice immunized and challenged with ovalbumin (ova) in a standard 21 day model. KB-R7943 abolished hyperresponsiveness to metacholine in this model (Figure 9), strongly suggesting an important role of NCX1 proteins in acute bronchoconstriction and hyperresponsiveness.

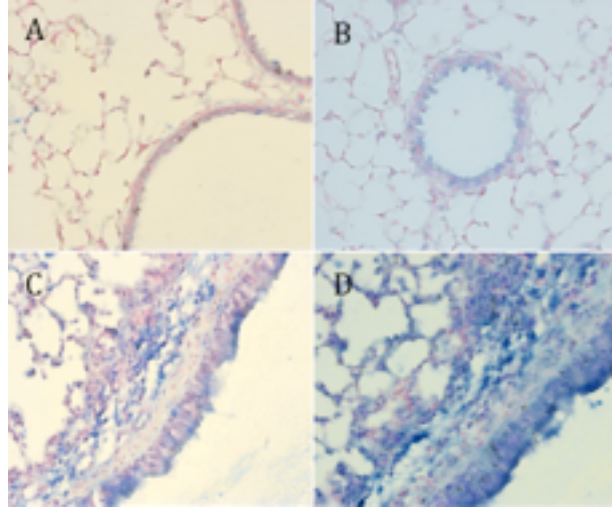


Figure 8. Immunohistochemistry for NCX1 in mouse lung. Normal human mouse lung (A,B) and mouse lung from ovalbumin model (C,D) were probed with either isotype control antibody (A,C) or an anti-NCX1 monoclonal antibody (R3F1)(B,D). Some background staining was observed, but specific epithelial staining was observed in both normal's and ovalbumin treated animals. This was increased in ovalbumin treated animals (D) and was evident in subepithelial smooth muscle.

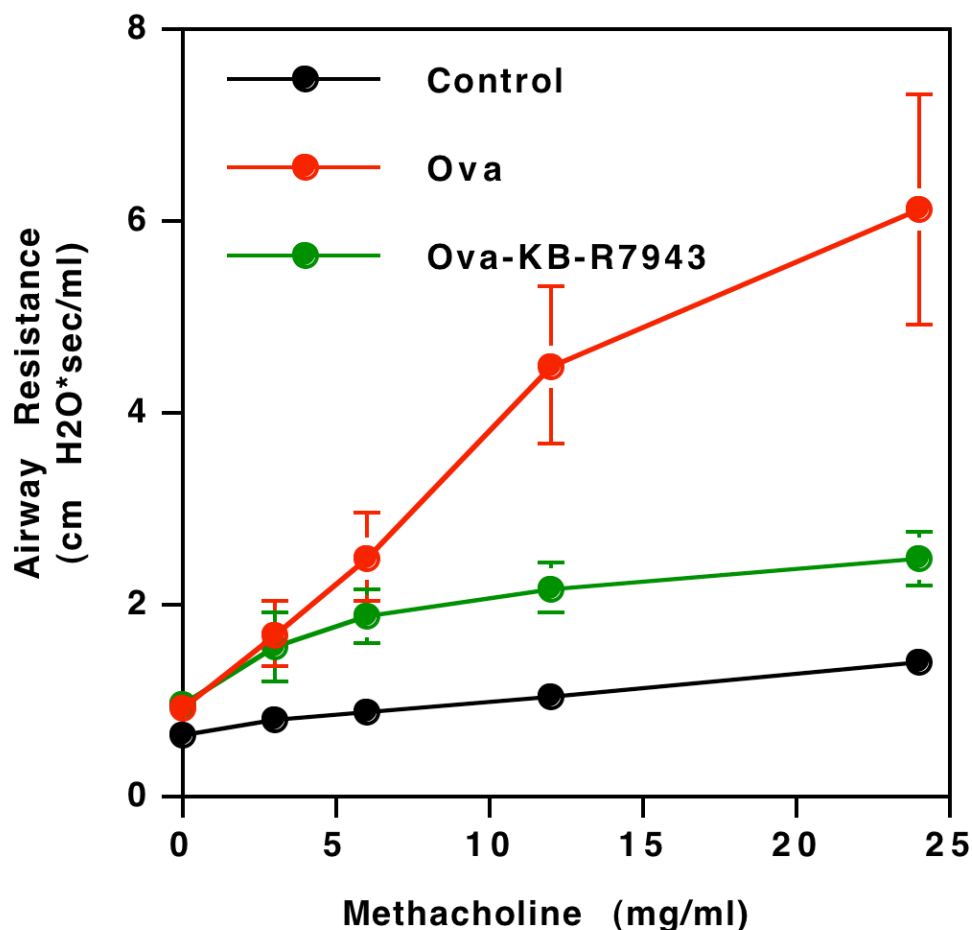


Figure 9. Inhibition of the reverse mode of NCX attenuates airway hyperresponsiveness in an ovalbumin model in mice. C57BL/6J mice were immunized and challenged with ovalbumin in a standard 21-day model. Thirty minutes before a methacholine dose-response curve mice received 10 mg/kg IP of KB-R7943 or vehicle. Control animals were also studied. $n \geq 4$ mice for each group.

Next the role of NCX1 proteins in controlling $[Ca^{2+}]_{cyt}$ homeostasis of HASMC's was studied. Western blots were done on HASMC's using R3F1, which recognized a protein in HASMC's with molecular masses of 70 and 120 kDa, corresponding to previous reports of the native NCX1 protein. Calcium flux in HASMC's was measured using fura 2-AM. $0 Na^+$ induced rise in $[Ca^{2+}]_{cyt}$ in HASMC's, and this rise was prevented using KB-R7943. This demonstrated that the reverse mode of NCX is active

in primary cultures of HASMC's. HASMC's were pretreated with $\text{TNF}\alpha$ and measured again using the previous method. $\text{TNF}\alpha$ enhanced activity of the reverse mode of NCX, which was prevented by KB-R7943 (Figure 10).

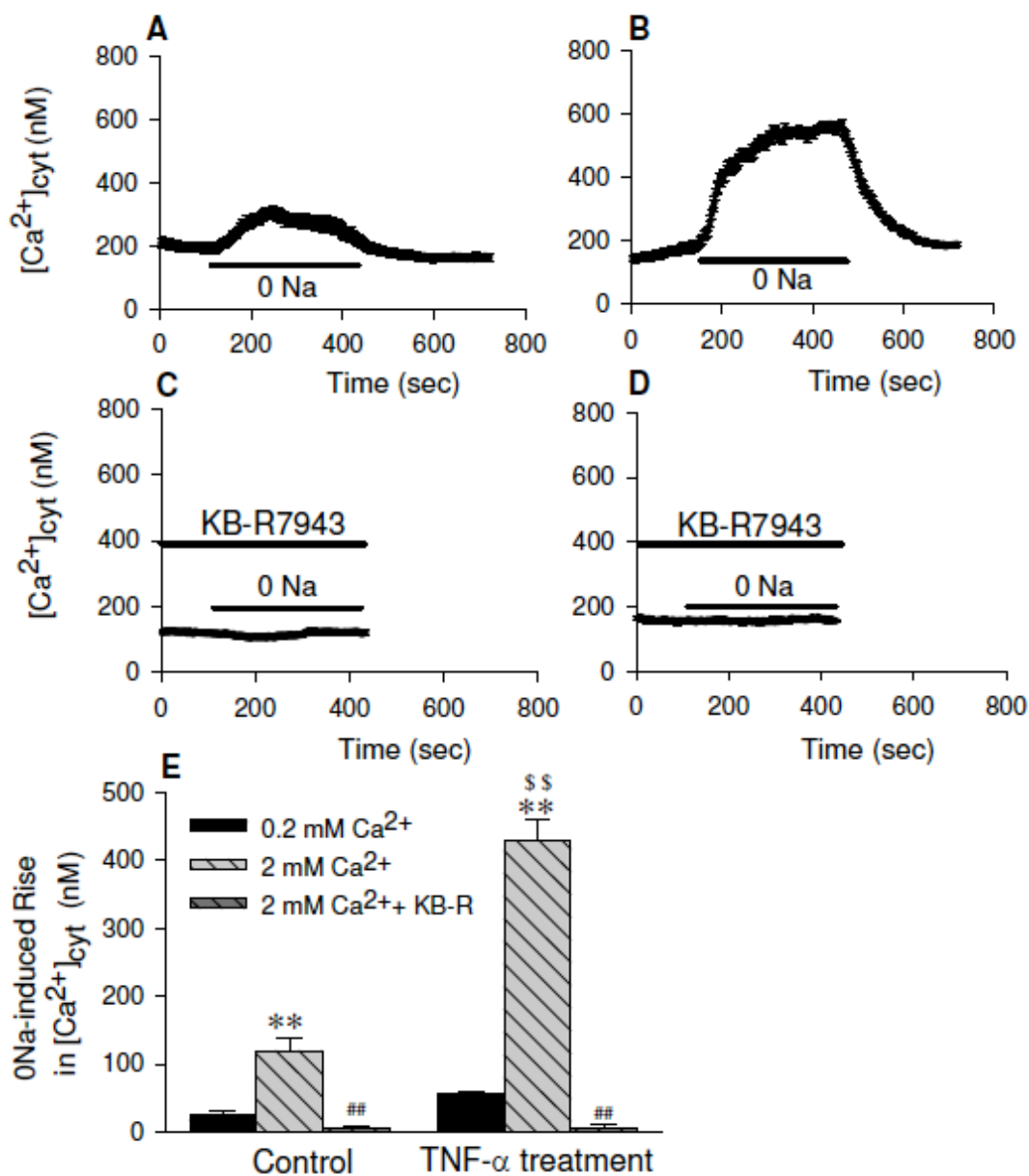


Figure 10. Expression and function of the reverse mode of NCX1 proteins and enhancement by $TNF\alpha$ in primary cultures of human ASM Cells. A) Removal of Na^+ induced $[Ca^{2+}]_{cyt}$ elevation in normal human ASM cells, which was prevented by KB-R7943 (30 μM). B) $0Na^+$ -induced $[Ca^{2+}]_{cyt}$ elevation was enhanced in human ASM cells pretreated with $TNF\alpha$ (20ng/ml) for 24h, which was again prevented by KB-R7943. E) Summarized data showing $0Na^+$ -induced $[Ca^{2+}]_{cyt}$ elevation in human ASM cells at different treatments. ** $P < 0.01$ vs. 0.2 $[Ca^{2+}]_{cyt}$; # $P < 0.001$ vs. 2 mM $[Ca^{2+}]_{cyt}$, \$\$ $P < 0.001$ vs. control. $N = 30-40$ cells for each group.

In summary, these preliminary studies demonstrate that $\text{Na}^+/\text{Ca}^{2+}$ exchangers exist and control $[\text{Ca}^{2+}]_{\text{cyt}}$ homeostasis and contraction of ASM in both animals and humans, and that $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity was enhanced by $\text{TNF}\alpha$ in HASMC's. The aims of this study are to determine whether or not inflammatory cytokines present in asthma induce airway remodeling, hyperreponsiveness, and inflammation via an NCX1 dependent mechanism.

Materials and Methods

I. Mice

Six-week old male C57BL/6 or BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed standard Teklad 7001 diet and were studied in accordance with NIH Guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committees of the University of California, San Diego and the VA San Diego Healthcare System.

II. Primary Cell Culture of Airway Smooth Muscle Cells

Human bronchial smooth muscle cells (HBSMCs) were purchased from ScienCell (Carlsbad, CA) and were cultured in smooth muscle growth medium (Lonza; Walkersville, MD), which consisted of smooth muscle basal medium, 5% FBS, 5 μ g/ml insulin, 2 ng/ml human fibroblast growth factor, and 0.5 ng/ml human epidermal growth factor, at 37° for 5 to 7 days before each experiment. Cells were plated on poly-L-lysine coated tissue culture flasks.

III. Colorimetric Proliferation Assay

HBSMC's were plated on poly-L-lysine coated 96-well tissue culture plates at 3×10^3 and serum-starved overnight. Cells were treated for 1-3 days. At each time point, 20 μ l of CellTiter 96 Aqueous One Solution (Promega, Madison, WI) was added to each well, then incubated at 37° for 1-h. Absorbance at 490nm was read on a 96-well plate reader following incubation.

IV. Small Interfering RNA Transfection

For siRNA experiments, a 19-mer siRNA was used to target NCX1 mRNA specifically (GenBank Accession NM_019268) with a sense strand (5'-3'): GGACCAAGAUGACGAGGAA from Invitrogen (Carlsbad, CA). This siRNA was screened against the GenBank database and no other significant matches were found. A scrambled sequence siRNA (Neg-siRNA) that had no sequence homology to any known genes was used as a control. Approximately 106 cells were used for siRNA transfection reaction. Before transfection, siRNA and Neg-siRNA were diluted with OPTI-MEM reduced serum medium and the mixture was mixed gently. This was followed by dilution of lipofectamine with Opti-MEM reduced serum medium. Lipofectamine was mixed with transfection solution. The Lipofectamine and siRNA mixture were then added to each culture well, and mixed. The cells were incubated in a humidified CO₂ incubator for 24 hours until the assay for gene knockdown is ready to be performed.

V. [Ca²⁺]_{cyt} Measurement with a Digital Imaging System

[Ca²⁺]_{cyt} in airway smooth muscle cells was measured by Fura-2 fluorescence ratio digital imaging as described previously (Dai, et al. 2006). Briefly, cells grown on cover slips were loaded with 5 μM Fura-2 acetoxymethyl ester (AM) (dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in PSS) at room temperature (22-24°C) for 50 min, then washed in normal PSS for 30 min. Thereafter, the cover slips with cells were mounted in a perfusion chamber on a Nikon microscope stage. Cells were initially superfused with PSS for 5 min at room temperature, and then switched to Ca²⁺-free or Na⁺-free solutions with different drugs. The ratio of Fura-2 fluorescence (510-nm light

emission excited by 340- and 380-nm illuminations) from the cells, as well as background fluorescence, was collected with the use of a 40x Nikon UV-Fluor objective and an intensified CCD camera (ICCD200). The fluorescence signals emitted from the cells were monitored continuously using a MetaFluor Imaging System (Universal Imaging, Corporation, Downingtown, PA, USA) and were recorded in an IBM-compatible computer for later analysis. $[Ca^{2+}]_{cyt}$ was calculated from Fura 2 fluorescent emission excited at 340 and 380 nm (F340/F380) using the ratio method based on the equation: $[Ca^{2+}]_{cyt} = Kd \times (Sf2/Sb2) \times (R - Rmin)/(Rmax - R)$, where Kd (225 nM) is the dissociation constant for Ca^{2+} , R is the measured fluorescence ratio, and $Rmin$ and $Rmax$ are minimal and maximal ratios, respectively (Grynkiewicz, et al. 1985).

VI. Western Blot Analysis of NCX1 Proteins

Proteins were extracted from mouse heart, lung and tracheal tissues, or from HBSMCs by homogenization on ice in 500 μ l of lysis buffer containing: 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM disodium EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, and complete protease inhibitor cocktail (AEBSF 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin 1.5 mM, and E-64 1.4 mM) Sigma, St. Louis, MO). Equal amounts of protein, as determined by Lowry assay (Dc assay; Bio-Rad, Hercules, CA), were combined with 2x Laemmli sample buffer. Samples were not boiled because this hydrophobic, membrane-bound protein was found to aggregate with boiling. Proteins were separated by electrophoresis on 4-15% SDS-PAGE and transblotted to nitrocellulose membranes. The protein-bound nitrocellulose membranes were first incubated 30 min at room temperature in blocking buffer containing 2% nonfat dry milk

in distilled water. Nitrocellulose membranes were incubated with R3F1 monoclonal antibody to NCX1 (Swant, Bellinzona, Switzerland) diluted in blocking buffer (1:5,000) overnight at 4°C and then rinsed for 1 h with a wash buffer containing 20 mM Tris, pH 7.5, 500 mM NaCl, and 1% Tween 20. The membranes were then incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG antibody for 30 min at room temperature and washed for 1 h with agitation, changing the wash buffer every 15 min. Protein bands were visualized with ECL Plus detection reagents (Amersham and Pharmacia, Piscataway, NJ), with NCX1 bands occurring at 120 and 70 kDa.

VII. RNA Extraction and Quantitative Reverse-Transcription PCR

Total cellular RNA was isolated from HBSMCs by the single-step guanidinium thiocyanate method using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To remove residual DNA, RNA samples were treated with RNase-free DNase and purity was confirmed by 260/280 ratios. RNA was reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA).

Real time PCR was performed in an Mx3000P real-time PCR system (STRATAGENE, La Jolla, CA, USA) using SYBR Green I dye as the detection format. A 16 µl reaction volume contained 16 ng of cDNA, 100 nM primers, using 1x of iQ Sybr Green super mix (Bio-Rad Laboratories; Hercules, CA). The primers for human NCX1 were (forward) TGTGCATCTCAGCAATGTCA and (reverse) TTCCTCGAGCTCCAGATGTT. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control (housekeeping gene) to control for potential differences in RNA input. The primers for GAPDH were (forward) ACAGTCAGCCGCATCTTCTT and (reverse) TGGAAGATGGTGATGGGATT. Data

were analyzed by determining the cycle threshold (Ct) and normalizing NCX1 Ct to GAPDH Ct using the $\Delta\Delta\text{Ct}$ method (Schmittgen, 2008).

VIII. Immunocytochemistry

HBSMCs were fixed in cyclohexylamine-formaldehyde fixative consisting of 0.45% (wt/vol) formaldehyde and (in mM) 75 cyclohexylamine, 75 NaCl, 10 EGTA, 10 MgCl_2 , and 10 PIPES. After fixation, the cells were permeabilized in fixative containing 0.5% polyoxyethylene 20 cetyl ether and were then incubated (4–17 h) in antibody buffer containing antibodies against NCX1 (clone R3F1, Swant). FITC-labeled donkey anti-mouse IgG was used to visualize the primary antibody. The fluorescence from the secondary antibody in the absence of primary antibody (positive control) did not exceed 2–3% of the fluorescence in the presence of antiserum.

IX. Mouse Model of Allergic Airway Inflammation

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) 6-8 weeks old were immunized and challenged over a 21 day protocol. On days 0 and 7 mice were immunized with intraperitoneal injections of 50 μg of LPS-free ovalbumin (Profos AG, Regensburg, Germany) adsorbed by 1 mg of alum (Sigma-Aldrich, St. Louis, MO) in 100 μl of PBS. Subsequently on days 17, 18, 19, and 20 mice were challenged with ovalbumin intratracheal instillations of 20 μg in 50 μl PBS delivered via direct visualization of the vocal cords and using a gel loading micropipette. On day 21, mice were sacrificed and lungs were homogenized for western blotting.

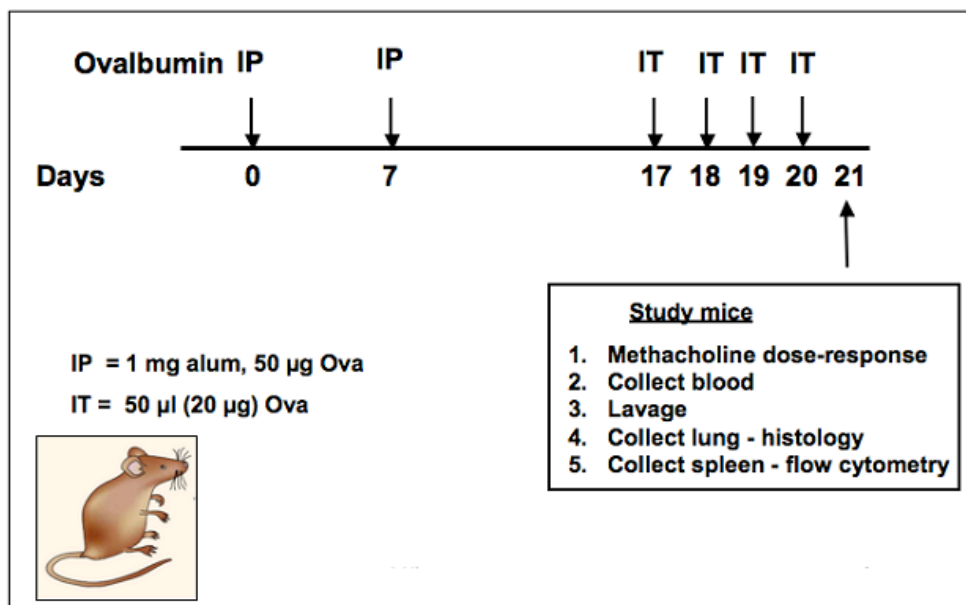


Figure 11. Ovalbumin model protocol. Immunization on days 0 and 7 were done by intraperitoneal injection of 50 µg ovalbumin and 1 mg alum. Challenge with 20 µg ovalbumin by direct intratracheal instillation was performed on days 17-20. Mice were studied on day 21.

Results

Feasibility of Proposed Techniques

Primary cultures of HBSMCs were plated on poly-L-lysine tissue culture plates and grown in smooth muscle media. They were grown to 70-80% confluence and treated with cytokines for experiments. When cell cultures reach 100% confluence, the smooth muscle cells detached due to tension. Therefore it was critical to work with cells before they reached 100% confluence.

Total mRNA was isolated from HBSMCs using TRIZOL. 1 μ g of total RNA was reverse transcribed by Superscript II reverse transcriptase (Invitrogen) using an oligo-(dT) primer. cDNA was analyzed using quantitative PCR. Initially, it was difficult to detect NCX1 signal, but by using RNase-free DNase to eliminate genomic DNA before the RT-PCR reaction, and by increasing the amount of cDNA in the qPCR reaction, we were able to obtain a better NCX1 message signal.

Total protein was isolated from HBSMCs using RIPA buffer. Proteins were diluted 1:2 with lamelli sample buffer and loaded onto 4-15% gradient gels (Bio-Rad). NCX1 was found to aggregate when heated, due to its hydrophobic nature. Therefore, proteins were slurred. Nitrocellulose membranes were found to bind NCX1 better than PVDF. Proteins were probed by primary mouse antibodies against NCX1 and GAPDH. Anti-mouse secondary antibodies were used to bind mouse primary antibodies and West Pico ECL solution was used to develop the blot.

HBSMCs were plated on 96-well tissue culture plates for colorimetric assays. Standard curves were generated on day zero. A concentration of 3×10^3 cells was found to be the optimum concentration for plating in 96-well plates. SN-6 was dissolved at a concentration of 10mM in 100% DMSO and diluted to 10 μ M in smooth muscle media.

Inflammatory Cytokines Increase NCX1 Expression in HBSMCs

The first set of experiments was designed to determine if inflammatory cytokines influence NCX1 expression in HBSMCs. In order to test the effect of inflammatory cytokines on mRNA encoding for NCX1, HBSMCs were grown to confluence and serum-starved for 24 hours. Complete media was subsequently added and HBSMCs were treated with 20 ng/ml of TNF α for varying periods of time. Extracted RNA subjected to real-time quantitative RT-PCR revealed that RNA encoding for NCX1 did not change in control samples over 6 h, but at 6 h, NCX1 mRNA in TNF α treated cells had significantly increased ~3.5-fold (Figure 12). GAPDH was used as a housekeeping gene and revealed no change.

To see if protein levels of NCX1 in HBSMC's were upregulated, cells were again conditioned with or without 20 ng/ml IL-33 and TNF α for 24 h and the proteins extracted and subjected to Western blotting. Bands corresponding to immunoreactive NCX1 were not denatured by heating because heating caused these hydrophobic proteins to aggregate. β actin was used as a loading control. NCX1 protein was significantly increased in the cells conditioned with IL-33 and TNF α and densitometry of these blots revealed a 2.5-

fold increase in NCX1 protein in the cytokine conditioned cells relative to controls (Figure 13). β actin bands showed equal loading in each well.

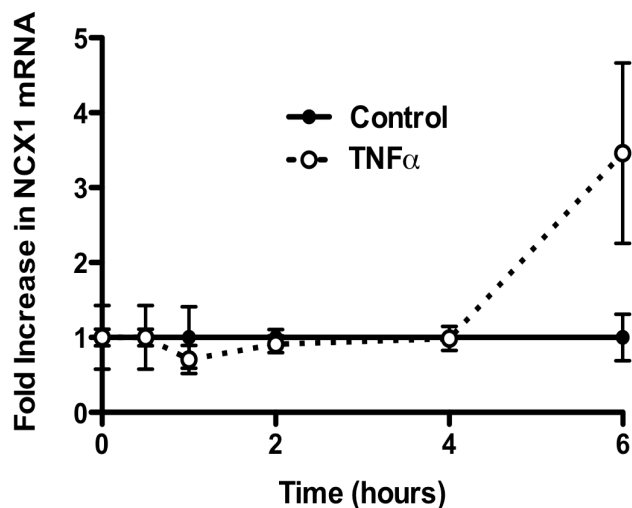


Figure 12. NCX1 mRNA is upregulated by TNF α treatment. HBSMC's were conditioned with or without 10ng/ml TNF α for up to 6 hours. RNA was extracted and analyzed by real time RT-PCR. Data were normalized to GAPDH. $P < 0.001$ vs. control, $n = 6$.

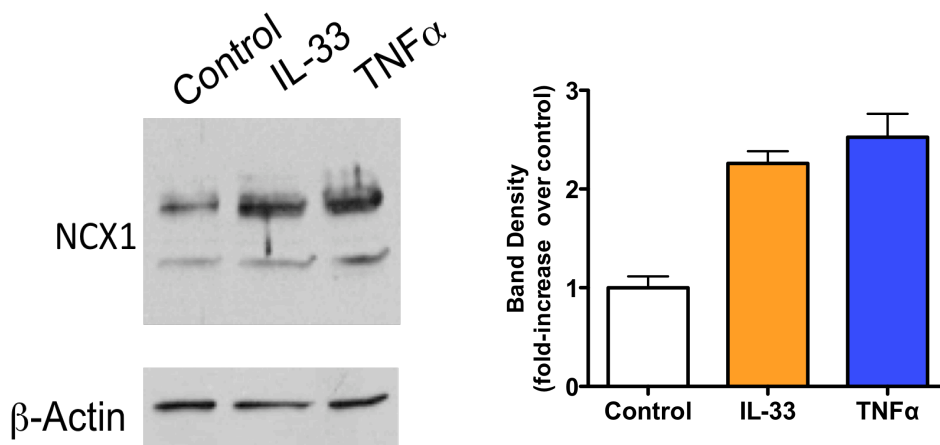


Figure 13. NCX1 protein is expressed in HBSMC and upregulated by cytokine. A representative blot of HBSMC's conditioned with or without 20 ng/ml of IL-33 or TNF α . Probed with the NCX1 monoclonal antibody R3F1. Densitometry analysis was performed with NIH Image J ($n = 5$, $p \leq 0.0001$).

To further examine the expression of NCX1 in HBSMC's, cells were again conditioned with or without 20 ng/ml IL-33 and TNF α for 24 h and the proteins extracted and subjected to immunocytochemistry. Modest amounts of NCX1 are present in HBSMC's at baseline that is increased by conditioning for 48h with 10 ng/ml of IL-13, IL-33, or TNF α (Figure 14).

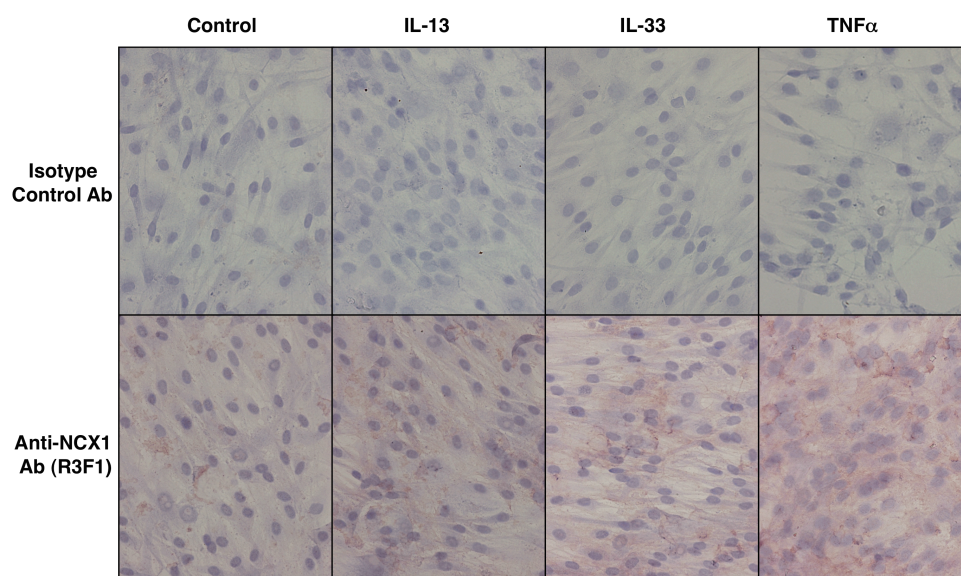


Figure 14. Immunocytochemistry demonstrates NCX-1 upregulation with cytokine treatment in HBSMC. Modest amounts of NCX1 are present in HBSMC's at baseline that is increased by conditioning for 48h with 10 ng/ml of IL-13, IL-33, or TNF α . (Chromogen is Vector Red, counterstain is hematoxylin, x400)

NCX1 Protein Expression in the Lungs of Ovalbumin-Treated Mice

To test *in vivo* regulation of NCX1, we used mice with ovalbumin induced asthma. A twenty-one day protocol of immunization with ovalbumin followed by airway challenge was developed in C57BL/6 mice (Figure 11). On day 21, the lungs were harvested and the proteins extracted and subjected to immunoblotting using R3F1 antibody directed against NCX1 (Figure 15). Densitometry was performed using NIH

Image J and NCX1 was normalized to β actin. NCX1 was substantially increased in ova treated mice compared to control. There was no change in β actin under the same conditions and probed on the same blot. These data demonstrate that NCX1 protein expression is increased in the lungs of ovalbumin-treated mice.

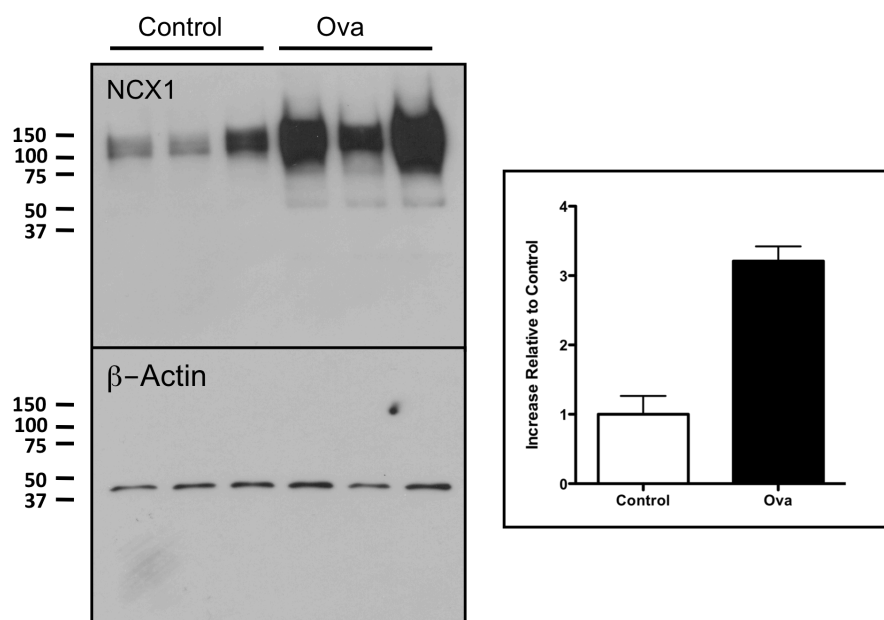


Figure 15. NCX1 protein expression is increased in the lungs of ovalbumin-treated mice. C57BL/6 mice were immunized and challenged with ovalbumin in a 21 d model. Controls received Pbs. On day 21, the lungs were harvested, proteins extracted, and subjected to immunoblotting with R3F1 antibody directed against NCX1. β actin was used as a loading control. Densitometry was performed using NIH Image J and NCX1 was normalized to β actin.

Functional Role of NCX1 in HBSMC Proliferation

Airway smooth muscle proliferation is a key component of airway remodeling and inflammation associated with asthma. In order to test whether or not asthma associated cytokines induced proliferation in airway smooth muscle, via NCX1, *in vitro* studies were done. HBSMC's were plated on poly-L-lysine coated 96 well tissue culture plates. 3000 cells were plated in each well and were serum-starved overnight using basal

smooth muscle cell media from SciencCell. Cells were given complete media and were subsequently treated with $\text{TNF}\alpha$, IL-13, and IL-33 at 20ng/mL. These treatments were also given in the presence of SN-6 (10 μM), a selective blocker of the reverse mode (calcium entry) of NCX1. HBSMC proliferation was measured at 24h, 48h, and 72h cytokine treatment using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. Significant increase in proliferation occurred in the presence of cytokine, compared to controls (Figure 16). Proliferation was blocked in the presence of SN-6 (Figure 17). These data obtained from the measurement of HBSMC proliferation provides support that asthma associated cytokines induce ASM proliferation, and that NCX1 plays a critical role in mediating this proliferation.

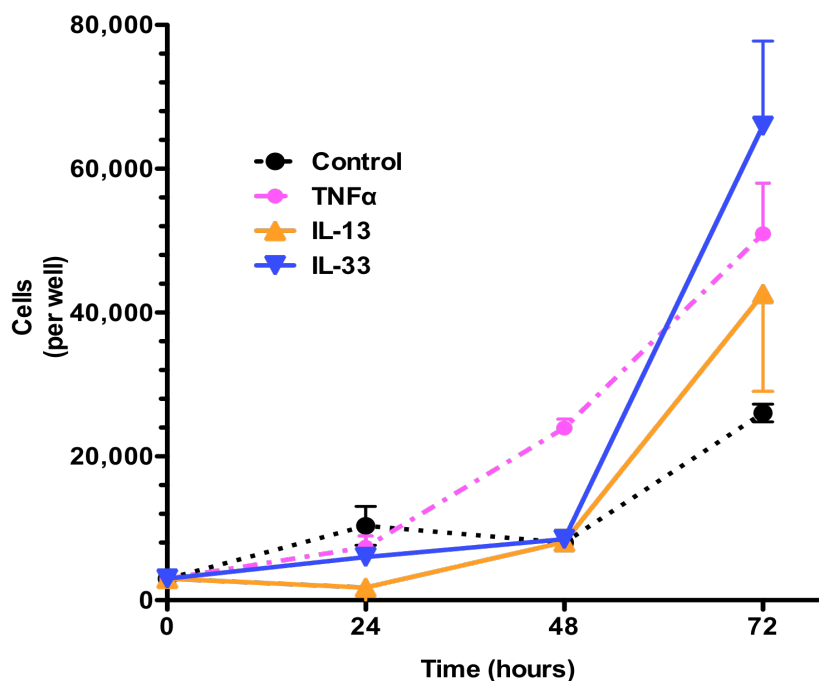


Figure 16. HBSMC proliferation is increased upon cytokine treatment. HBSMC, obtained by endobronchial biopsy in patients undergoing bronchoscopy, were conditioned with cytokines at 20 ng/ml up to 72h. The cells proliferate in response to $\text{TNF}\alpha$, IL-13, and IL-33. 3000 cells were plated in each well of a 96-well plate. ($n = 5$, $p \leq 0.01$ **, $p \leq 0.001$ ***)

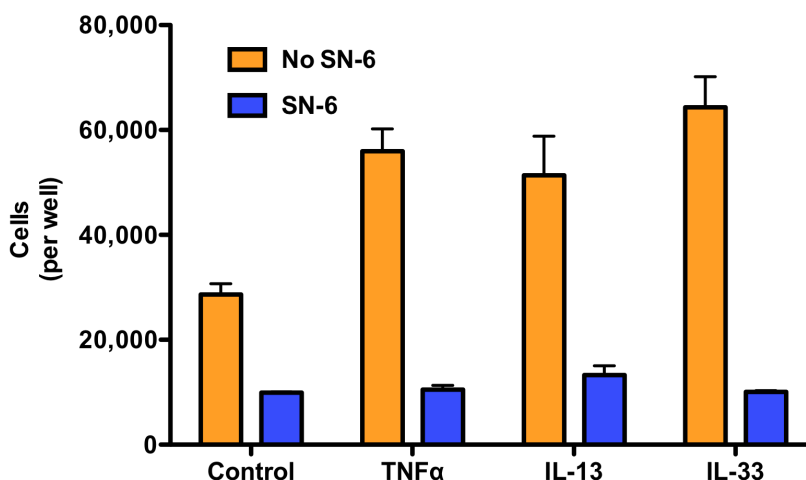


Figure 17. HBSMC proliferation is blocked with NCX1 inhibitor at 72h treatment. HBSMC proliferation was blocked by adding the inhibitor of the reverse mode of NCX1, SN-6. (n=6, $p \leq 0.001$ ***)

Identification of NCX1 with a siRNA Knockdown Approach in HBSMCs

To further test the expression and function of NCX1 in HBSMCs, we performed siRNA experiments. NCX1 mRNA was detected by RT-PCR in Neg-siRNA-transfected HBSMCs (Figure 18). However, after the cells were transfected with NCX1 siRNA for 24 hours at increasing concentrations (100 and 200 nM), NCX1 mRNA expression decreased in a dose-dependent manner. Cells were loaded with fura 2-AM and then $[Ca^{2+}]_{\text{cyt}}$ in single cells was determined with a digital Ca^{2+} imaging system. Basal $[Ca^{2+}]_{\text{cyt}}$ and $0Na^+$ -induced $[Ca^{2+}]_{\text{cyt}}$ were measured simultaneously in HBSMCs with Neg-siRNA or NCX1 siRNA transfection (200 nM) for 24 hours. $0Na^+$ -induced $[Ca^{2+}]_{\text{cyt}}$ was significantly attenuated in NCX1 siRNA transfected cells compared with Neg-siRNA-transfected cells although basal $[Ca^{2+}]_{\text{cyt}}$ was similar between them (Figure 18). These data show that NCX1 is important in maintaining $[Ca^{2+}]_{\text{cyt}}$ in HBSMCs.

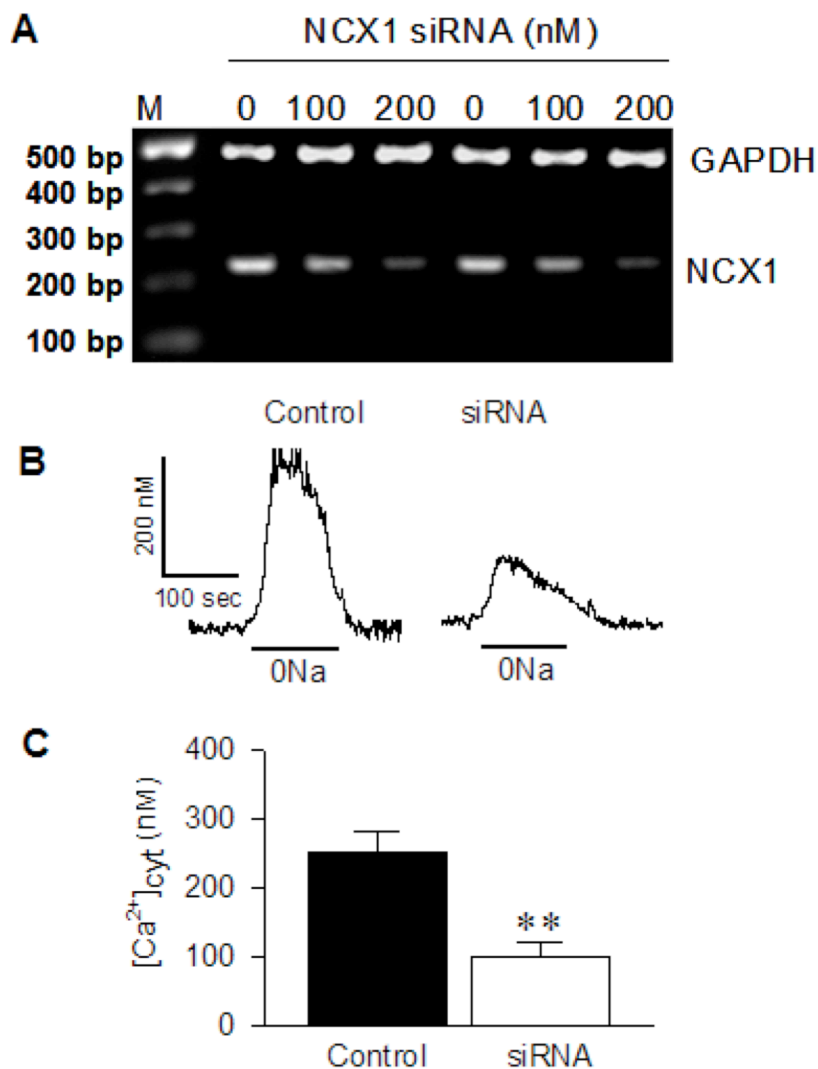


Figure 18. siRNA knockdown of NCX1 decreases function as measured by calcium flux.

A) HBSMCs were plated on cover-slips 24 h, they were transfected with Neg-siRNA (N) or NCX1 siRNA for 24 h. They were then lysed to extract total RNA. RT-PCR was performed to reveal mRNA expression of NCX1. B) After HBSMCs on cover-slips were transfected with Neg-siRNA (Control) or NCX1 siRNA (200 nM) for 24 h, they were loaded with fura 2-AM and then 0Na⁺ induced [Ca²⁺]_{cyt} in the cells was measured by digital Ca²⁺ imaging system. C) Summary showing 0Na⁺ induced [Ca²⁺]_{cyt} elevation in HBSMCs with Neg-siRNA (Control) or NCX1 siRNA transfection. **P<0.01 vs. control, n=20-30 cells

Discussion

HBSMC's treated with inflammatory cytokines showed increased expression of NCX1 and increased proliferation compared to control. Moreover, proliferation was blocked using SN-6, a specific inhibitor of the Ca^{2+} entry mode of NCX1 (Kita, et al. 2007). Additionally, siRNA knockdown of NCX1 decreased 0Na^+ induced calcium flux in HBSMC's. Finally, NCX1 was found to be upregulated in lungs from asthmatic mice compared to control. These findings suggest that abnormal Ca^{2+} homeostasis associated with increased NCX1 expression contributes to airway remodeling in asthma.

Increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in airway smooth muscle has a variety of effects such as contraction, proliferation, hypertrophy, and airway remodeling, all of which contribute to airway hyperresponsiveness (Berridge, 2006; Orrenius, et al. 2003). $[\text{Ca}^{2+}]_{\text{cyt}}$ is governed by plasma membrane ion channels and exchangers (Hirota, 2007). Attempts to decrease $[\text{Ca}^{2+}]_{\text{cyt}}$ by blocking voltage-gated Ca^{2+} channels using currently available Ca^{2+} channel blockers failed as therapeutic agents for the treatment of asthma (Barnes, 1998). Subsequent studies indicated that in airway smooth muscle, voltage-independent pathways, such as TRP channels and $\text{Na}^+/\text{Ca}^{2+}$ exchangers, make greater contributions to $[\text{Ca}^{2+}]_{\text{cyt}}$ homeostasis. TRP channels have been studied extensively in airway smooth muscle, but the asthma field has neglected $\text{Na}^+/\text{Ca}^{2+}$ exchangers. Since TRPC3 and NCX1 were shown to be functionally coupled (Eder, 2007), a recent report that $\text{TNF}\alpha$ enhances TRPC3 in airway smooth muscle cells prompted us to study the role of NCX1 in airway smooth muscle (White, et al. 2006).

Our finding that NCX1 is upregulated in airway smooth muscle in response to inflammatory cytokines coincides with the previous reports regarding TRP channels. They strengthen the claim that TRPC3 and NCX1 may be functionally coupled. However, more studies will be done using different combinations of cytokines. A recent study demonstrated synergism between cytokines (Prefontaine, 2009). Multiple cytokines are released simultaneously during inflammatory asthma (Barrios, 2006). Using combinations of cytokines may provide a better representation of what takes place during asthma. Further experiments will also be conducted to test NCX1 expression in airway smooth muscle obtained from mild to severe asthmatic patients versus healthy patients.

In addition to *in vitro* studies, *in vivo* studies were conducted to study the regulation of NCX1 in asthmatic mice. Lung homogenates from ova-treated mice had substantially increased NCX-1 protein mass compared to controls. This suggests that NCX1 plays an important role in asthma. Although NCX1 expression in ova-treated mice was substantially increased, lung homogenates contain many different cell types and increased NCX1 expression may be from tissues other than airway smooth muscle. Further studies will be conducted to test the localization of NCX1.

Our findings that SN-6 blocks HBSMC proliferation and that siRNA knockdown of NCX1 blocks calcium entry into HBSMCs coincide with an earlier study by Trian and colleagues. They suggest that altered calcium homeostasis in smooth muscle leads to increased mitochondrial biogenesis, which enhances smooth muscle proliferation (Trian,

et al. 2007). Our findings suggest that NCX1 contributes to $[Ca^{2+}]_{cyt}$, which then controls airway smooth muscle proliferation.

Overall this study demonstrates that total NCX1 protein expression is increased *in vitro* in response to inflammatory cytokines present in asthma, and *in vivo* in ova treated mice. Increased NCX1 expression leading to abnormal calcium homeostasis in cytokine treated HBSMC's was associated with increased proliferative function. Blockade of NCX1 translation using siRNA resulted in decreased calcium flux. These results imply that targeting of NCX1 may be a potential therapeutic route for treating asthma. Though, further experiments should be conducted on smooth muscle obtained from asthmatic patients. These studies also raise a new question for further research: is it elevated $[Ca^{2+}]_{cyt}$ resulting from increased NCX1 that contributes to airway hyperresponsiveness and remodeling, or is increased NCX1 expression a marker of more proximal signaling changes induced by inflammation?

Greater attention to calcium homeostasis has revealed that increased $[Ca^{2+}]_{cyt}$ in airway smooth muscle has a variety of effects such as contraction, proliferation, and airway remodeling, all of which contribute to asthma. This new understanding of calcium homeostasis has generated interest regarding what contributes to increased $[Ca^{2+}]_{cyt}$. In addition to plasma membrane calcium channels, the SR has calcium channels that have recently received much attention. The sarco/endoplasmic reticulum $[Ca^{2+}]$ (SERCA) pump reuptakes $[Ca^{2+}]$ into the sarcoplasmic reticulum after a rise in $[Ca^{2+}]_{cyt}$, and was recently found to be downregulated in airway smooth muscle obtained from asthma patients (Mahn, 2009). Downregulation of SERCA causes a slower

reuptake of calcium into the SR, resulting in increased $[Ca^{2+}]_{cyt}$. This raises new questions regarding the relationship between SERCA and NCX1: Do SERCA and NCX1 influence each other's expression or are they both markers of more upstream signaling changes induced by inflammation? Future research will be done to shed light on this question. This paper discussed the role of NCX1 mediated regulation of $[Ca^{2+}]_{cyt}$ in airway smooth muscle. As discussed, NCX1 may be a potential target for the development of treatments against asthma and airway inflammation. This study has illuminated NCX1's function in airway smooth muscle, but more work must be done to confirm the significance of NCX1 in airway smooth muscle from asthmatic patients.

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