QATAR UNIVERSITY

COLLEGE OF PHARMACY

CATHEPSIN B INDUCED CARDIOMYOCYTE HYPERTROPHY REQUIRES ACTIVATION OF THE Na^+/H^+ EXCHANGER ISOFORM 1

BY

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ABSTRACT

Multiple studies have demonstrated that proteases, specifically cathepsin B (Cat B) and matrix metalloproteinase-9 (MMP-9) contribute to the remodeling of the extracellular matrix (ECM), a hallmark of cardiac hypertrophy (CH). Cat B is activated under acidic conditions, a key stimuli of the Na^+/H^+ exchanger isoform-1 (NHE1). Enhanced NHE1 expression/activity have also been demonstrated to contribute to the progression of CH. Whether NHE1 contributes to the Cat B hypertrophic response remains unclear. NHE1 activity was stimulated in H9c2 cardiomyoblasts using 10 μM Angiotensin (Ang) II ±10 μM EMD, a NHE1 inhibitor or 10 μM CA-074Me, a Cat B inhibitor and characterized for changes in the cell surface area, protein content and atrial natriuretic peptide (ANP) mRNA, indices of cardiomyocyte hypertrophy. The localization of Cat B in lysosomes was measured using LysoTracker Red dye. The release of Cat B from the intracellular to the extracellular space was assessed by measuring Cat B protein expression in the media. MMP-9 was also measured in the extracellular space and assessed for its contribution to the CAT B hypertrophic response. NHE1 increased the Cat B protein (136.56 ± 9.4% Ang II vs. 100% control, 37 kDa and 169.84 ± 14.24% Ang II vs. 100% control, 25 kDa; $P<0.05$) and gene expression (288.11 ± 76.72% Ang II vs. 100% control; $P<0.05$) and induced cardiomyocyte hypertrophy. Furthermore, Cat B protein expression and MMP-9 activity were increased in the extracellular space. These effects was regressed upon inhibition of NHE1 or Cat B. Our study demonstrates for the first time that Cat B is involved in the NHE1 mediated cardiomyocyte hypertrophic response in cooperation with the activation of MMP-9.

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ABBREVIATIONS

AE, Anion exchanger

Ang II, Angiotensin II

ANP, Atrial natriuretic peptide

BCECF-AM, 2'-7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester

Bcl-2, B-cell lymphoma-2

Bax, Bcl-2 Associated X protein

BNP, β-natriuretic peptide

CaM, Calmodulin

CaMK II, Ca^{2+}/Calmodulin-dependent protein kinase II

CaN/NFAT, Calcineurin/nuclear factor of activated T cells

Cat, Cathepsin

CH, Cardiac hypertrophy

CHE, Cl^-/OH^- exchanger

CVDs, Cardiovascular diseases

DCM, Dilated cardiomyopathy

DMA, 5-(N, N-Dimethyl) amiloride hydrochloride

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, Dimethyl sulfoxide

E64, L-trans-Epoxysuccinyl-leucylamido (4-guanidino) butane

ECM, Extracellular matrix

EIPA, 5-(N-ethyl-N-isopropyl) amiloride

EMD, N-(2-methyl-4,5-bis(methylsulfonyl)-benzoyl)-guanidine hydrochloride

ER, Endoplasmic reticulum

ERK Extracellular signal-regulated kinase

ET-1, Endothelin-1

FBS, Fetal bovine serum

GPCR, G protein coupled receptors

GSK-3β, Glycogen synthase kinase-3β kinase

IGF, Insulin like growth factor

IL-1β, Interleukin-1β

LC3, Microtubule associated protein light chain 3

MAPK, Mitogen activated protein kinase

MCT, Monocarboxylate transporters

Me, Methyl ester

MHC, β-myosin heavy chain

MI, Myocardial infarction

MMPs, Matrix metalloproteinases

mRNA, messenger ribonucleic acid

MT-MMPs, Membrane-type metalloproteinases

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

M6P, Mannose-6 residue phosphorylation

NBC, Na^+/HCO_3^- co transporter

NCX, Na^+/Ca^{2+} exchanger

NF-kB, Nuclear factor kappa B

NHE1, Na^+/H^+ exchanger isoform 1

NKA, Na^+/K^+ ATPase

OPN, Osteopontin

PE, Phenylephrine

PI3Kα, Phosphatidylinositol 3 kinase alpha

PKB/Akt, Protein kinase B/Akt

RIPA, Radioimmunoprecipitation assay

SEM, Standard error of the mean

UPS, Ubiquitin proteasome system

α1-AR, α1 adrenergic receptor

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DEDICATION

To all those in a never ending pursuit for knowledge

Chapter 1: INTRODUCTION

1.1 Cardiac Remodeling

The World Health Organization predicts that by 2030, 23.3 million people can die of cardiovascular pathologies with heart failure being the leading cause of death amongst them, with the highest increase occurring in the Middle East (1, 2). Over the past three decades, the national burden of disease in the population of Qatar has shifted dramatically. A recent study has shown that ischemic heart disease is a primary cause of death in Qatar (3). Hence, heart failure is a serious health problem that needs to be addressed immediately. The key pathophysiological process that ultimately leads to heart failure is cardiac remodeling which occurs in response to various cardiovascular pathologies such as cardiac hypertrophy (CH), hypertension, and myocardial infarction (MI) if left untreated (4, 5). Cardiac remodeling occurs as a result of unbalanced and uncontrolled activity of extracellular matrix (ECM) components. Myocardial remodeling is characterized by intrinsic changes of the cardiomyocyte and the interstitium such as reorganization of myocytes, increased collagen deposition, increased fibrosis, changes in intercellular matrix components as well as enlargement of the cardiomyocytes, and is one of the major mechanisms that lead the heart to failure (3, 4). Components of the ECM including cathepsin (Cat) cysteine proteases and matrix metalloproteinases (MMPs) have a central role in ECM remodeling and have been implicated in the development and progression of cardiovascular diseases (CVDs). Activation of extracellular proteases and the degradation of the ECM, factors which contribute to the remodeling of the ECM, have been associated with decreased extracellular pH. A decreased extracellular pH is induced upon Na^+/H^+

exchanger isoform 1 (NHE1) activation. The cellular interplay of components of the ECM and the activation of NHE1 in the setting of cardiac remodeling is the focus of this project. CH, one of the hall marks of heart failure, occurs as a result of uncontrolled ECM remodeling. Hence, knowledge about its underlying pathological process may hold a key to developing therapeutic strategies.

1.2 Cardiac Hypertrophy

CH is primarily characterized by the enlargement of the cardiomyocytes. It can occur as a result of increased work load, hormonal stimuli, and various chronic diseases like hypertension (6). CH can be differentiated into physiological or pathological hypertrophy. Factors such as increased physical exercise and pregnancy can cause physiological hypertrophy of the heart (7, 8). While pathological hypertrophy can be caused by increased blood pressure and enhanced neurohormonal activation (9). Pathological hypertrophy, if left untreated can eventually lead to heart failure (10). Physiological and pathological hypertrophy can be distinguished by the structural and molecular changes occurring. Pathological hypertrophy entails the activation of fetal genes such as B-type natriuretic peptide (BNP), atrial natriuretic peptide (ANP) and α-actin and β-myosin heavy chain (MHC). In addition, an increase in cell size, protein synthesis, and increased fibrosis are usually seen in pathological hypertrophy (11, 12). Physiological hypertrophy generally does not induce the activation of hypertrophic markers and does not increase the accumulation of myocardial collagen (13, 14). Understanding the underlying mechanisms of CH is of vital importance in terms of finding potential therapeutic targets.

1.2.1 Mechanisms of Cardiac Hypertrophy

Multiple intracellular signaling pathways regulate CH. Physiological hypertrophy causes the activation of protein kinase B (AKT), the insulin like growth factor (IGF), and phosphatidylinositol 3 kinase alpha (PI3Kα) pathways (15). While pathological hypertrophy could be activated by the activation the calcineurin/nuclear factor of activated T-cells (CaN/NFAT), the mitogen activated protein kinases (MAPKs) pathway, cardiac specific membrane proteins, integral components of the ECM as well as G-protein coupled receptors (GPCRs) and receptor tyrosine kinases have also been implicated in contributing to pathological hypertrophy. GPCRs are widely implicated and extensively studied in relation to CVDs making them important targets for treatment of cardiac diseases (16). It is well known that GPCRs can be activated by binding of agonists such as angiotensin (Ang) II, catecholamines and endothelin-1 (ET-1). The activation of GPCRs leads to signaling via different G protein sub types ($G\alpha_s$, $G\alpha_q$, $G\alpha_i$, $G_{\beta\gamma}$) and couples to MAPKs and phospholipase C or, in the case of β-adrenergic receptors, to adenyl cyclase and protein kinase A (17-19). It has been demonstrated that over expression of cardiac specific $G\alpha_s$ sub unit in transgenic mice induced CH, necrosis and fibrosis upon stimulation with catecholamines (20, 21). This suggests that the GPCR signaling pathway plays a major role in the cardiac hypertrophic response and could hold the key to its prevention and treatment.

1.3 Extracellular Matrix

The ECM is composed of protein such as collagens, elastin, proteoglycans, glycoproteins and proteolytic enzymes which are responsible for the maintaining the ECM

structure. The ECM functions to form a 3D network amongst the cells and tissues and maintains the integrity of the extracellular compartment. The ECM is highly dynamic and is constantly undergoing changes in order to facilitate processes such as cell proliferation, differentiation, and angiogenesis (22).

Proteolytic activation and subsequent degradation of the ECM proteins are amongst the most important maintenance processes in the ECM. Proteases such as cathepsins and MMPs can cause alteration in the ECM structure. This can result in the release of biologically active molecules, which influence growth, migration, adhesion, and pathological processes (23). MMPs and serine proteases are primarily localized extracellularly and are active at neutral pH, hence they were traditionally thought to be the main players mediating extracellular proteolysis (24, 25). Whereas, lysosomal proteases like the cathepsins were thought to be the majorly involved in intracellular protein turnover due to their requirement of an acidic pH for optimal enzyme activity. However, it has been recently been discovered that the cathepsins can be released under pathological conditions whereby they can remain active in the extracellular compartment and mediate ECM degradation (26-30).

1.3.1 ECM and the Cardiac System

The ECM is a critical component of the cardiovascular system. Controlled synthesis and degradation of ECM proteins is essential for maintaining the physiological state and functioning of the cardiovascular system. However, under pathological conditions such as CH and hypertension, there seems to be disturbances in the ECM. These disturbances seem to arise due to uncontrolled activities of proteases such as cathepsins and MMPs (31). Imbalance in the ECM has been shown to be the cause of many pathological conditions such as CVDs, tumors, osteoporosis. Hence, knowing the physiological functioning and the pathological changes that occur in the ECM may hold a key to unveiling underlying mechanisms.

Cardiac remodeling occurs primarily as a result of abnormal activation of proteases such as cathepsins and MMPs, which results in an imbalance between the synthesis and degradation of ECM proteins, resulting in uncontrolled ECM remodeling. (32-35). A study reported that the levels of Cat S and K increased significantly with increasing elastolytic activity in the tissue extracts from the failing rat myocardium; this response was blunted by the broad spectrum cysteine protease inhibitor or a specific inhibitor of Cat S (36, 37). Moreover, active Cat S, K, and L have been shown to degrade ECM proteins, including laminin (38), fibronectin (39), elastin (40), and collagens (41, 42). Activation of MMPs has also been reported to cause left ventricular remodeling in hypertensive heart failure rats (43). It is well established that the various isoforms of MMPs can degrade majority of the ECM proteins such as various types of collagen, gelatin, fibronectin and laminin (44-47). Moreover, cathepsins have the ability to activate pro-MMPs as well. Together, the evidence

suggests that enhanced activation of cathepsins and MMPs causes uncontrolled ECM remodeling which results in various cardiovascular pathologies (48, 49). Much has already been established however, there are still significant void in the understanding their role in the failing heart.

One of the ECM proteins, osteopontin (OPN), was recently shown to be elevated during CH (50). Moreover, transgenic mice expressing the cardiac specific active Na^+/H^+ exchanger isoform 1 (NHE1) showed up regulation of OPN mRNA expression in the heart compared to the control (50). It was recently shown that the activation or over expression of NHE1 causes the up regulation of OPN in cardiomyocytes, which contributes towards CH (51, 52). Moreover, NHE1 has been independently shown to induce CH in multiple *in vitro* and *in vivo* models (53-55) (discussed in section 1.5.3.3).

Another ECM protein that has been shown to interact with OPN and also to contribute towards cardiac remodeling is CD44 (56). It is found on the cell-surface and is involved in cell–cell interactions, cell adhesion and migration. The CD44 is responsible for transducing intracellular signals in the myocardial cells (57). Moreover, a study reported increased expression of CD44 in the hypertrophic heart of aorta-ligated rats. Another study reported up regulation of CD44 in infarcted mice hearts as compared to control (58). From the above mentioned reports it seems that the components of the ECM play an important role in the cardiac remodeling processes, hence the need to further investigate them. Proteases such as cathepsins and MMPs have been suggested to play a major role in causing an imbalance in the synthesis and degradation of the ECM. Hence, understanding them may hold a key to understanding the underlying pathological processes.

1.4 Proteases

Proteases represent about 1 to 4 % of the genes per genome that have been sequenced till date and are found in all living forms (59). They are enzymes that are involved in the catabolism of unwanted or damaged proteins by hydrolyzing the peptide bonds between the amino acids that form them. The hydrolysis of the peptide bonds can be executed as a result of the endo and exopeptidase activity of the proteases. Besides their primary role in the turnover of proteins, proteases have been identified to play a central role in many pathological process that are involved ECM degradation and cardiac remodeling. Amongst all proteases, cathepsins and MMPs are amongst the most extensively studied proteases in cardiovascular disease and remodeling.

1.4.1 Classification of Proteases

Proteases have been classified based upon their catalytic site or based upon the homology of their amino acids sequences or based upon the homology of their 3D structure (60). The human genome consists of about 670 proteases which can be classified based upon their site of action into MMPs (constitute about 33% of total proteases), serine (31%), cysteine (25%), aspartic (4%), threonine, and glutamic protease (59, 60). Since Cat B contains a cysteine residue within its catalytic site, it will fall under the cysteine class of proteases. Proteases can also be classified based upon the homology of their amino acid sequences to a representative member. Papain, a plant protease, has high homology to Cat B. Hence, Cat B is the reference protease of the papain family. Other proteases that are also

part of the papain like family are Cat C, F, H, K, L, O, S, V, W, and X (60). Proteases are also classified by the homology of their 3D structure and the arrangement of their residue on the catalytic site. Cat B belongs to the CA clan because of its specific arrangement with the cysteine and histidine residue on the active site (60).

1.4.1.1 Cathepsins

Cathepsin is derived from the Greek word *katehepsin* which means to digest. Cysteine proteases, discovered in the second half of the 20th century, are the largest sub family of cathepsins. They are ubiquitously expressed in all cell types and can be primarily found within the lysosomes, where they function to remove damaged and unwanted proteins. Cathepsins have been shown to be expressed by cardiomyocytes (36), macrophages (61), and immune cells (62).

1.4.1.1.1 Isoforms and Classification of Cathepsins

Cathepsins comprise of eleven different isoforms (Cat B, C, L, F, H, K, O, S, V, W, and X) (27). Cathepsins are further divided into endopeptidases (Cat F, K, L, S, and V) or (62) exopeptidases (Cat B, C, H, and X) based upon their cleavages sites (26, 59, 63). Most proteases have either endopeptidase or exopeptidase activity. However, Cat B is unique in this respect as it has the ability to have both activities along with having peptidyl dipeptidase (64) and carboxypeptidase (65) activities (59, 60). This unique property of Cat B has been attributed to a particular element in its structure called the occluding loop (66). At low pH, the occluding loop covers the active site hence limiting access of its active site

to polypeptides. This limits the enzyme's endopeptidase activity but retains its dipeptidase and carboxypeptidase activities (67). At physiological pH, the loop uncovers the active site thereby granting more access to the enzyme's active site. This allows for the endopeptidase activity of the enzyme (67). Owing to Cat B's wide range of peptidase activities, it is capable of degrading a wide range of proteins. However, this makes Cat B an even dangerous protease under abnormal or pathological condition.

1.4.1.1.2 Structure and Synthesis of Cathepsins

Cathepsins have been shown to be composed of three general domains, a single peptide, pro region, and a catalytic domain (consisting of a heavy chain, and a light chain) (27, 68-70) (Figure 1.1). The single peptide, located at the N-terminal of the enzyme, is about 10 to 20 amino acids in length and is responsible for the translocation of the enzymes into the endoplasmic reticulum (ER) during messenger ribonucleic acid (mRNA) translation (28). The cathepsins are then moved to the acidic compartments of lysosomes or endosomes, through either the mannose-6-phosphate (M6P) receptor dependent or independent pathways. Here, the pro region is cleaved off which results in the liberation of the fully active form of the cathepsin which functions to get rid of unwanted proteins (71-73). Moreover, the acidic compartments give the lysosomal cathepsins the optimum pH for their activity. In the case of Cat B, the pro region can be cleaved off by Cat D (aspartic protease) and also by self-cleavage (74-76). The pro region of different cathepsins can fall anywhere between 36 (Cat X) to 271 (Cat F) amino acids in length. The catalytic domain contains the heavy and the light chain of the enzyme and can fall anywhere between 241

to 260 amino acids in length. The catalytic domain contains the active site of the enzyme which is composed of a cysteine, a histidine, and an asparagine residue (Figure 1.1). The pro and the active forms of cathepsins can also be secreted into the extracellular compartment by exocytosis (77) (Figure 1.1).

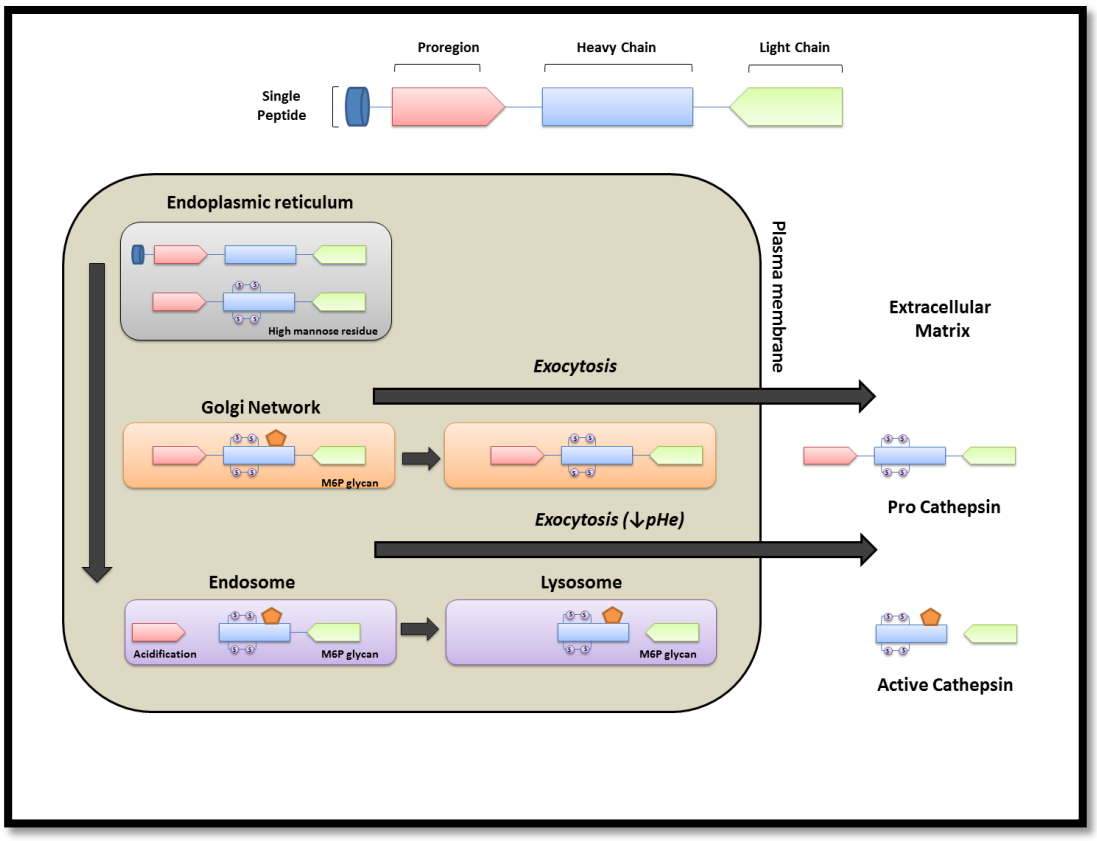


Figure 1.1 A model of the cathepsin primary structure and cathepsin maturation processes. A. Cathepsins contains a signaling peptide, pro region, heavy chain, and light chain. B. The cathepsins maturation process is as follows: The single peptide is removed in the ER and disulfide-bound formation, followed by glycosylation of the M6P residue and translocation into the Golgi network. The cathepsins are then translocated into the endosomes or lysosomes where the pro region is cleaved off. The Ca^{2+}-mediated organelle fusion and secretion into extracellular spaces can also take place from the endosomes or lysosomes. ER, endoplasmic reticulum; M6P, mannose-6 residue phosphorylation.

1.4.1.1.3 Physiological Role of Cathepsins

Gene ablation studies have revealed that cathepsins have varied physiological functions which are important for many biological processes.. Their functions can be at most times defined by their tissue localization as in the case of Cat F, S, and K (42, 78-80) or general functions such as protein degradation as in the case of ubiquitously expressed Cat B, H, and L (81). Cat S and Cat L are involved in the processing of the MHC class II-associated invariant chain (78, 82). Cat K, found primarily within the osteoclasts, has been shown in multiple studies to be an important player in normal bone remodeling processes (78). Cat F, found in the macrophages, has recently been suggested to be an important player in the atherosclerotic process and remodeling of the intimal ECM (61). Lysosomal Cat, B, H and L, are primarily involved in the bulk protein degradation of proteins with in the lysosomes (27, 83, 84). Lysosomal cathepsins are also involved in the conversion of pro hormones into their active forms by cleavage of their pro peptides (85). Disturbance in the normal functioning of the cathepsins are thought to result in ECM imbalance and in turn pathological conditions such as CH (79). Cathepsins also participate in apoptosis, although the mechanisms are not yet clearly defined (26).

1.4.1.1.4 Regulation of Cathepsins

Cathepsins can be regulated in a number of ways, two of the most important being through pH changes and inhibition by their endogenous inhibitors, the cystatins (11). Cathepsins are primarily regulated by pH when they are localized within the lysosomes.

However, once they are released from the lysosomes into the cytosol or into the extracellular compartment, their activity is regulated by the cystatins.

Regulation by pH changes

Generally lysosomal cathepsins are optimally active at slightly acidic pH, around pH 5,such as those found within the lysosomes (86). The lysosomes are membrane bound compartments which contain an acidic microenvironment. The acidic environment is created and maintained by pumps that are present on the lysosomal membranes called H^+-ATPase pumps. These pumps pump in H^+ using ATP into the lysosomes thereby maintaining an acidic environment of about pH 5. This provides the cathepsins with an optimal pH to function. The low pH also creates a reducing environments within the lysosomes. This prevents the oxidation of the thiol group on the active site of the cathepsins and hence prevents their inactivation (59). The pH and redox status-dependent activity of cathepsins thus limits their proteolytic efficacy to the endosomal/lysosomal compartment. However, it is to be noted that not all cathepsins are optimally active at an acidic pH. Some cathepsins like Cat S have the ability to be enzymatically active at neutral pH as well (59).

The activity of Cat B has been demonstrated to be optimal at an acidic pH value and reduced at neutral pH range (87). Cat B also shows changes with respect to its endo and exopeptidase activity based upon changes in pH. Cat B shows dominant endopeptidase activity at lower pH values whereas it shows more dominant exopeptidase activity at higher pH values. This has been attributed to a special element in its structure called the occluding loop. At low pH, the occluding loop covers the active site hence limiting access of its active

site to polypeptides. This limits the enzyme's endopeptidase activity but retains its dipeptidase and carboxypeptidase activities (67). At physiological pH, the loop uncovers the active site thereby granting more access to the enzyme's active site. This allows for the endopeptidase activity of the enzyme (67). Nonetheless, it has been found that Cat B still retains significant proteolytic activity at neutral pH as well (88).

Regulation by endogenous inhibitors

Cystatins are further divided into intracellular cystatins (stefins A and B), extracellular cystatins (cystatin C), and kininogens whereby they control the activity of cathepsins under physiological conditions. They are competitive, reversible inhibitors of the cathepsins. They inhibit the activity of the cathepsins by binding on to the active site thereby blocking access for the substrates and hence inhibiting their activity. The cathepsin inhibitors important to maintain the necessary balance during physiological conditions. However, imbalance between the cathepsins and cystatin levels has been reported under pathological conditions. Decreased levels of cystatin C have been reported in human atherosclerosis and aortic aneurysm (89). Moreover, higher cystatin C levels have been associated with having a higher risk of CVDs (90). Hence, they are also thought to be important biomarkers for CVDs (90).

1.4.1.1.5 Cathepsins in Cardiovascular Pathologies

Cathepsins have been shown to be important mediators in many cardiac pathologies. Previous studies have demonstrated that the various isoforms of cathepsins mediate cardiac pathologies in varied ways. Some isoforms have been reported to play a cardiotoxic role, whereas others have been shown to be cardioprotective.

Cathepsins in Cardiac Hypertrophy and Heart Failure

Hypertension refers to enhanced arteriole pressure and total peripheral artery resistance as a result of hemodynamic overload on the heart. It is well established that high blood pressure causes CH, fibrosis, remodeling, and heart failure of Cat S and/or K gene and protein levels which were increased in the failing rat myocardium in association with hypertension, (36). The levels of the Cat S, B, and K genes have also been shown to be increased whereas those of cystatin C showed no significant changes in Dahl salt sensitive rats, a model of hypertension (36). Immunohistochemical analysis revealed the expression of Cat S and K in the myocardium of hypertensive heart failure rats was markedly increased as compared to control rats (36). Furthermore, the elastase assays demonstrated that elastase activity of Cat S and K was increased significantly in the tissue extracts from the failing rat myocardium. This response was blunted by the broad spectrum cysteine protease inhibitor E64 or a specific inhibitor of Cat S (36, 37). Similar to the findings in the rat model, the amounts of Cat S and K were found to be increased in the failing myocardium of patients with hypertensive heart failure (36). Importantly, Qing Wu *et al* hypothesized that Cat B could also play a prominent role in the pathology of pressure overload-induced

cardiac remodeling. Wu *et al* showed that Cat B levels were upregulated in cardiomyocytes in response to hypertrophic stimuli both *in vivo* and *in vitro*. Moreover, knockout of Cat B attenuated pressure overload-induced CH, fibrosis, dysfunction, and apoptosis. These results were further confirmed in *in vitro* studies utilizing H9c2 cardiomyocytes in which hypertrophy was induced by Ang II. Pharmacological Cat B inhibition using CA-074Me suppressed the cardiomyocytes hypertrophy by inhibiting the ASK1/JNK pathway. Although, the exact mechanism by which Cat B acts in cardiac diseases is still unclear, these data indicate an important role of Cat B in the modulation of cardiomyocyte hypertrophic response by regulating the signaling pathways involved in cardiac remodeling. Taken together, it seem that there is an interplay between the various forms of cathepsins in CH and heart failure. More studies are needed to elucidate the role each cathepsin plays in the settings of CH and heart failure.

Cathepsins in Cardiomyopathy

Cardiomyopathy refers to the deterioration of the heart muscles to contract, and usually precedes heart failure. In humans, Cat B mRNA and protein levels were shown to be greater in dilated cardiomyopathy (DCM) than in control hearts (91). Levels of Cat S, B, L, and/or K were also shown to be increased in subjects with dilated and hypertrophic cardiomyopathies compared with control subjects (77). Moreover, genetic studies have revealed that Cat L deficient mice developed interstitial myocardial fibrosis, a characteristic sign of cardiomyopathy (92, 93).Taken together, it seems that various cathepsins are involved in the processes that contribute towards cardiomyopathy.

Cathepsins in Myocardial Infarction

Cat B, L, and H were the first to be found in the rat myocardial response to acute MI (94). It has been shown that Cat B protein levels and activity in cardiac tissues changed in response to injury (95). Another recent study reported an increase in Cat B levels post-MI *in vivo*. Cat B inhibition, using specific pharmacologic inhibitor CA-074Me, significantly attenuated cardiac dysfunction, and reduced cardiomyocyte size and cardiac fibrosis in the experimental MI model, by inhibiting NLRP3 activation (96). It was demonstrated in two hypertensive heart failure models (aortic banding and Ang II infusion) that the human Cat L transgenic heart shows a decrease in overload-induced CH and fibrosis through blocking of the AKT/GSK3 signaling pathway (97). Moreover, in post infarction cardiac repair, deletion of Cat L reduced the expression of angiogenic factors and decreased revascularization activity (98). Forced expression of Cat L in mature endothelial cells considerably enhanced their invasiveness and increased their angiogenic capacity *in vivo* (99). Moreover, it was demonstrated *in vivo* that myocardial cystatin C is increased in mice that develop heart failure in response to hypoxic injury and that this increase is associated with local inhibition of Cat B activity and accumulation of collagen and fibronectin (100). A study reported that MI induced by left coronary artery ligation in wild-type rats caused rapid Cat L activation in myocardium and bone marrow and its deficiency contributed to diminished function and adverse remodeling late post-MI (98). This study showed that cardiac repair and remodeling benefits from activation of Cat L to improve cardiac function after MI injury (98). Moreover, Cat S seems to play a role in

cardiac maintenance as well since its deletion exacerbated angiotensin (Ang) II induced cardiac inflammation (101). Evidence suggests that cathepsins play diverse role in the CVS where some act as cardioprotective while others play a more cardiotoxic role. More studies are needed to elucidate the role of the various cathepsins in CVDs.

Cathepsins in Coronary Artery Disease

ECM remodeling, including collagen and elastin degradation by extracellular proteases, contributes to wall stiffening and valve dysfunction (102, 103). Interstitial cells in mitral valves express excessive levels of proteolytic enzymes such as Cat S and K, as well as MMP-1 and -13, suggesting a role of cathepsins in valve disease (104). Human stenotic aortic valves have been shown to contain much greater amounts of Cat S, K, and V mRNAs and proteins than controls. (105). Recent *ex vivo* work demonstrates that cyclic stretch increases Cat S, K, and L in porcine valves, accelerating the destruction of aortic valvular ECM and the progression of aortic stenosis (106). Cat S deficiency lowered the arterial and aortic valve calcification in ApoE deficient mice (106). Taken together, it seems that various cathepsins are intimately involved in the pathological processes contributing to coronary artery disease.

Cathepsins in Atherosclerosis

It was reported that human macrophages secreted active Cat S, B, and L that exhibited elastolytic activity (107). More recently, both the gene and protein levels of these enzymes were found to be increased in murine diet-induced atherosclerotic plaques (108). Cat S and K were the first cysteine cathepsins found to show increased protein levels in human atherosclerotic plaque (89, 109). Increased expression of cathepsins occurs in macrophages bordering the lipid core adjacent to the fibrous cap and in macrophages and smooth muscle cells in the regions surrounding atherosclerotic plaques (110, 111). Evidence suggests that cathepsins play a major role in the pathogenesis of atherosclerosis. It has become clear that both intracellular and extracellular activities can cause the mechanisms of action of cathepsins in atherosclerosis (112).

1.4.1.1.6 Cathepsin and Apoptosis

Apoptosis is known to be one of the main processes that contribute the heart to failure in progression to CH and cardiac remodeling, (113, 114). Apoptosis is a programmed cell death mechanism which when activated leads to biochemical and cellular changes which eventually terminate in cell death. A family of 14 cysteine proteases called caspases primarily regulate apoptosis (115). Apoptosis can occur via an extrinsic pathway or an intrinsic one. Extrinsic pathways is mediated by activation of death receptors whereas, the intrinsic is mitochondrial- mediated. Both of these pathway can activate caspases which then degrade various polypeptides in the cell including major structural elements, deoxyribonucleic acid (DNA) repair machinery, and protein kinases (115). The intrinsic

pathways plays a major role in cardiomyocyte apoptosis (116), primarily through Bcl-2 protein and procaspase activation (117, 118). Previous reports have demonstrated that increased caspase 3 caused a fall in left ventricular function and inhibition of this caspase attenuated ventricular remodeling (119-121).

The role of cathepsins in pathological apoptosis in cardiac disease has been investigated in several animal studies (122). Previous studies have demonstrated that cathepsins are essential for cardiomyocyte apoptosis (97). Cat B has been shown to be closely related to apoptosis (123, 124). An absence of Cats B and L has been shown to induce neuronal loss and brain atrophy (125). Cat B has also been shown to mediate caspase-independent cell death in non-small cell lung cancer cells (126). Once Cat B is released from the lysosomes into the cytoplasm it may activate or enhance apoptotic pathways (126). Moreover, the anti-apoptotic molecules Bcl-2, Bcl-xL, Mcl-1, and XLAP (X-chromosome linked inhibitor of apoptosis) are targeted by the lysosomal Cat B and L in several human cancer cell lines (127). Taken together, it seems that cathepsins, specifically Cat B, play an important role in mediating the processes that lead to apoptosis.

1.4.1.1.7 Cathepsin in the Autophagy-Lysosomal Pathway

The autophagy–lysosomal system and the ubiquitin–proteasome system (UPS) are the primary pathways that maintain the protein turnover in eukaryotic cells (128). In the cardiovascular system there is a fine balance between the two systems to maintain homeostasis of proteins and organelles (129). The UPS targets small and short lived protein by tagging them with ubiquitin (129). In contrast, the autophagy–lysosomal pathway

targets long-lived bulky proteins that are defected or damaged under stress (130). Dysregulated protein degradation, which can be caused by abnormal or enhanced activation of cathepsins and MMPs, has been demonstrated to contribute to cardiac diseases (131).

Cathepsins are important lysosomal protein–processing enzyme and have previously been demonstrated to be important in maintaining the autophagy- lysosomal pathway activated in response to stress (131). A study reported that the deficiency of lysosomal Cat L resulted in the enhanced activation of UPS in the setting of ischemia (131). The final step of the autophagy–lysosomal pathway is the fusion of an autophagosome with a functioning lysosome. An imbalance of protein homeostasis by dysfunction of this system may lead to pathological hypertrophy. Decreased extracellular pH values, which can be caused by an overactive NHE1 (132, 133), can cause increased redistribution of lysosomes to the cell surface (86, 134, 135) which may be accompanied by fusion with an autophagosome (131). Subsequently, the lysosomal contents can be secreted into the ECM as it has been shown for Cat B (86). A study demonstrated that extracellular acidic pH values caused the redistribution of the lysosome to the cell periphery which paralleled Cat B secretion. Interestingly this effect was blocked with several broad and specific NHE inhibitors (136). However, the role of the autophagy-lysosomal pathway in mediating the hypertrophic cascade in relation to Cat B and NHE1 has not been investigated and remains unknown.

1.4.1.1.8 Cathepsin Inhibitors

The discovery of L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) was seminal to the development of cathepsin inhibitors. E64 is a non-selective inhibitor of the papain family and works by covalently binding onto the active site by alkylation. E64 is an important inhibitor because it selectively inhibits only the papain family of proteases (137). An important derivative of E64 is E64d. E64d is an ethyl ester prodrug, which is hydrolyzed in the gut to its active form E64c which is more potent (60). Another derivative of E64 is the CA-074 which is a selective Cat B inhibitor. It functions by irreversibly binding on to the active site and thereby inhibiting with high potency. CA-074Me was developed from CA-074 to have better membrane permeability but it has the ability to inhibit both Cat B and L (60). Another cathepsin inhibitor is Leupeptin. Its non-selectively inhibits the cysteine and serine proteases by covalently binding on to the active site. Due to its decreased selectivity for the papain family of proteases, it is less widely used that E64 (137).

1.4.1.1.9 Cathepsins Inhibitors in Clinical Trials

A few cathepsin inhibitors are now being investigated in human trials for several diseases such as osteoporosis and rheumatoid arthritis. At least four different Cat K inhibitors have entered clinical trials for the treatment of osteoporosis and skeletal disorders with excessive bone remodeling. One compound, odanacatib, presently in phase III clinical testing (138). Cat S inhibitors, are in preclinical trials for the treatment of

rheumatoid arthritis (30). One Cat B inhibitor has completed Phase 1 trials for fatty liver disease (139) and another is in late preclinical stage for treating Chagas disease (140). However, none of the compounds have been examined for their effect in the setting of cardiovascular diseases.

1.4.1.1.10 Clinical Relevance

A previous study demonstrated that the levels of Cat B protein expression were elevated in myocardial samples obtained from DCM patients as compared to controls (91). Moreover the gene expression of Cat B was also shown to be elevated (91). Hence, inhibition of Cat B might prove to be beneficial for the prevention and treatment of DCM. Moreover, a study demonstrated that Cat B knockout mice maintained their health and were showed similar effects as normal littermates in behavior, histology, and fertility (141). The normal health of mice lacking Cat B implicates that pharmacologic inhibition of Cat B can potentially prove to be beneficial in treating cardiomyopathies.

1.4.1.2 Matrix Metalloproteinases

MMPs are a group of proteolytic enzymes that constitute over 20 isoforms. They are primarily responsible for the maintenance of the ECM. Apart from maintenance of the ECM they also take part biological processes, such as degradation of connective tissue, morphogenesis, angiogenesis, growth, and wound healing (47, 142). MMPs play an important role in the pathogenesis of various disorders (143). They are also known to be integral to the processes that cause cardiac remodeling and in turn hear failure (144).

Therefore, understanding the role of MMPs in physiological and pathological conditions is crucial to unveiling the underlying mechanisms of implicated diseases.

1.4.1.2.1 Isoforms and Classification of MMPs

MMPs have the ability to cleave a wide variety of ECM substrates. Each isoform has its own ECM substrate specificity. MMPs are classified into 6 distinct categories based upon their target substrates. They are collagenases, stromelysins, matrilysins, gelatinases, membrane-type metalloproteinases (MT-MMPs) and other MMPs, namely zinc- and calcium-dependent endopeptidases (45) (Figure 1.2). MMP-1, -8, -13 constitute the collagenases family and are responsible for breaking down collagen type I, II, and III at neutral pH (145, 146). The gelatinases family include MMP-2 and MMP-9. In particular gelatinases break down gelatin, denatured collagen, type IV, intact collagen of basal membranes and also non-denatured collagens type V, VII, X, XIV, fibronectin, agrecan, and elastin (147). Stromelysins include MMP-3, MMP-10, and MMP-11 have a wide substrate specificity. They are responsible for the degradation of all non-collagen ECM proteins such as proteoglycans, glycoproteins, fibronectin, and laminin (148). Macrophagous elastase, MMP-12, have the ability to break down elastin. Gelatinases and matrilysins are also able to degrade elastin along with fibronectin, laminin, basal membrane collagen, entactin, and chondrotin sulfate (149). MMPs are able to degrade all of the ECM components with their combined activities

1.4.1.2.2 Structure of MMPs

MMPs are synthesized as preproenzymes. The pre region is cleaved off to allow for the secretion of the inactive proenzymes from the cell into the ECM whereby they are activated by other proteases or matrix components. A proenzyme consists of 3 general domain: N-terminal propeptide, catalytic domain, and the C-terminal domain (Figure 1.2). N-terminal propeptide consists of about 80–90 amino acids containing cysteine residue. The cysteine residues interacts with the catalytic zinc through its side chain thiol group, and this ensures the inactivity of the proenzyme. Cleavage of a highly conserved sequence present in the propeptide region causes activation of the zymogene (150, 151).

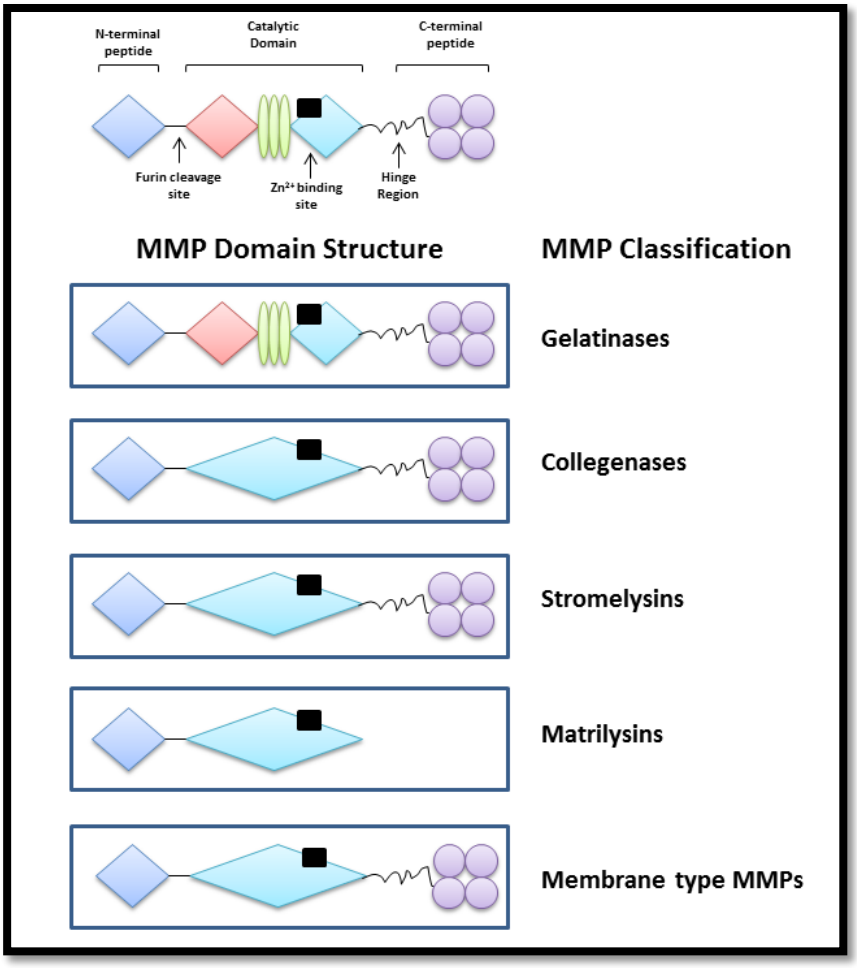


Figure 1.2 MMP domain structure and classification. A proenzyme molecule is organized into the 3 basic structural domains: N-terminal propeptide, catalytic domain, and the C-terminal part of the molecule. MMPs are classified into gelatinases, collagenases, stromelysins, matrilysins, and membrane type MMPs. MMP, matrix metalloproteinases

1.4.1.2.3 Regulation of MMPs

The regulation of MMPs activity includes multiple levels of activation of pro MMPs, inhibition, secretion of the enzyme molecule and the regulation of gene transcription or inhibition of MMPs activity by tissue inhibitors of metalloproteinases (TIMPs). The regulation of MMPs activity includes various levels of activation of latent

MMPs, inhibition by tissue inhibitors of metalloproteinases (TIMPs), secretion of the enzyme molecule and the regulation of gene transcription.

Regulation of MMPs by Cysteine Switch Mechanism

After propeptide cleavage, the MMP zymogens are changed into active enzymes (152). Propeptide contains a conservative sequence, which maintains the enzyme in latent form by the chelating action of cysteine residuum to Zn^{2+} ions at the catalytic site of the enzyme (150). Activation of the enzyme is explained by a so called mechanism of a cysteine switch (150). The key step is dissociation of zinc-cysteine interaction. Extracellular activation of the enzyme includes 2 steps. The first is initial cleavage of the MMP propeptide by protease, destabilization of propeptide binding interactions, and break of coordination bond of cysteine and Zn^{2+} ions. The second step is the final cleavage of the propeptide that is usually ensured by another MMP. The result is a matured enzyme (153, 154).

Inhibition of MMPs By Means of TIMPs

TIMPs are the main endogenous regulators of MMPs in the tissue and are responsible for maintaining the balance between the synthesis and degradation of MMPs (155). TIMPs molecular weight fall in the range of 21–30 kDa. They constitute four isoforms namely, TIMP-1, -2, -3, -4 (155). They bind to MMPs in a ratio 1:1 forming binary non-covalent complexes. In this way they protect binding sites for the substrates

being split off (155). TIMP-1 forms the complex preferentially with MMP-9, and TIMP-2 preferentially with MMP-2 (155).

1.4.1.2.4 MMPs in Cardiovascular Pathologies

Multiple lines of evidence suggest that enhanced levels of vasoactive molecules in the circulation can lead to abnormal or enhanced activation of MMPs (144). These molecular changes cause the preload and afterload on the heart to increase, thereby causing changes in the cardiac structure as a means to compensate for the enhanced workload. (144). The compensatory cardiac remodeling is thought to be one of the factors that leads the heart to failure. Under normal physiological conditions, the MMPs are responsible for the degradation of misfolded and unwanted proteins, thereby maintaining the ECM structure (34, 156). However, under pathological conditions there is abnormal activation of MMPs, which leads to uncontrolled ECM remodeling and thereby causing various cardiac diseases (33, 34). Although, the role of MMPs in cardiac diseases has been investigated for quite some time now there are still significant voids in understanding the underlying mechanism in the failing heart.

MMPs in Hypertension and Cardiac Hypertrophy

Hypertension refers to elevated pressure in the arteries and total peripheral resistance as a result of hemodynamic overload on the heart. Chronic increase in blood pressure is known to be one of the causes of cardiac remodeling, CH, and heart failure (5). Studies have demonstrated increase in MMP-9 protein expression and activity whereas, those of

TIMP-4 were reduced in Dahl salt-sensitive rats, a model of hypertension (157). In Dahl salt-sensitive rats, the gene expression and MMP-2 and MMP-9 activity were shown to be upregulated prior to the occurrence of left ventricular dilatation, systolic dysfunction, and pulmonary edema. Moreover, angiotensin converting enzyme inhibitors were able to abrogate cardiac remodeling suggestion a link between hypertensive hormone stimuli and proteolytic remodeling (158). Taken together, the findings from both hypertensive animal models and hypertensive patients suggest that various MMPs may be involved in the development of hypertensive cardiomyopathies.

CH, a hallmark of heart failure, involves changes in the ECM structure such as increased collagen deposition, increased fibrosis and increase in the cardiomyocyte size (159). Increased MMP-9 and MMP-2 expression have also been associated with increased heart weight and cardiomyocyte area (160). Serum levels of both MMP-2 and MMP-9 have been shown to be increased in patients with hypertrophic cardiomyopathy. However, only MMP-9 levels were associated with fibrosis (44). It has also been reported that increased MMP-2 and MMP-9 levels in CH are associated with a significant imbalance between MMPs and their endogenous inhibitors, TIMPs. Although both extracellular and intracellular modifications by proteases are known to occur for obtaining the hypertrophic phenotype, mechanisms of these processes are not fully understood.

MMPs in Dilated Cardiomyopathy

DCM is a heart-muscle disorder that can lead to decreased functioning of the heart. DCM if left untreated eventually leads to heart failure. DCM is characterized by a distended myocardium, increased interstitial fibrosis, chamber dilatation, and impaired functioning of the cardiac contractile muscles (161). The activities of various proteases have been demonstrated to vary depending upon the stage of the development of DCM. In general, MMPs activities appear to contribute to the development of both MI-induced and idiopathic DCM as a result of their extensive ECM remodeling capability. MMP-1 and MMP-13 are able to degrade both intact collagen and proteoglycans which are further altered by MMP-2 and MMP-9 (162). In a DCM rat model, increased levels of MMP-2, MMP-9, and TIMP-1 mRNA were reported at 2 weeks post DCM induction (163). However, as the DCM progressed into its 6^{th} week, only the level of MMP-2 mRNA were seen to be elevated. The results from this study imply that in DCM, MMP-9 plays a role in disease progression during the early stages, whereas MMP-2 remains involved for the entire developmental stages of DCM (163). Another study reported that MMP-9 protein levels were increased, whereas the increase in MMP-2 activity was normalized at the end of an 8-week period post-aortic banding (164). Although there are discrepancies in MMPs activities between these two experimental models, it is clear that differential activation of specific MMPs results in the progression of cardiac remodeling. In addition, increased levels of MMP-2, MMP- 3, and MMP-9 have been observed in patients with DCM (165-167). It is clear from the results that various form of MMPs are involve in the processes of cardiac remodeling.

1.5 Intracellular pH Regulation in the Heart

Multiple physiological process such as cell growth, cell volume regulation, differentiation, cell migration require strict control of pH in mammalian cells. Many enzyme and cell metabolic processes are rely upon the maintenance of optimal pH for their functioning.

1.5.1 Mechanisms of Intracellular pH Regulation

The intracellular pH is maintained by multiple mechanisms. The NHE is the primary pH-regulator in mammalian cells (168, 169). The NHE family of pH regulators maintains pH by exchanging intracellular protons (H^+) for extracellular sodium ion (Na^+) in a stoichiometric ration of 1:1, hence protecting the cell from acidosis. The NHE family is also involved in cell volume regulation by the exchange of H^+, Na^+, Cl^- in and out of the cell (169). Thus, NHE is an important role in maintaining the pH and cellular metabolism in mammalian cells. In the myocardium, maintenance of the concentration of various ions like protons, sodium and calcium is of critical importance for normal functions like cell excitability, contractility, and cell volume. Five types of pH regulators have been identified in the myocardium, out of which two function to remove protons while three of them function to bring in protons and thereby protect the cell against acidosis or alkalosis (reviewed in (170, 171) (Figure 1.3). Amongst all the pH regulators, the NHE1 plays the most important role in pH maintenance as it is responsible for 50-60% of the proton efflux (172). Thus, understanding the role of NHE1 is of vital importance in order to expose the underlying mechanisms of cardiac diseases.

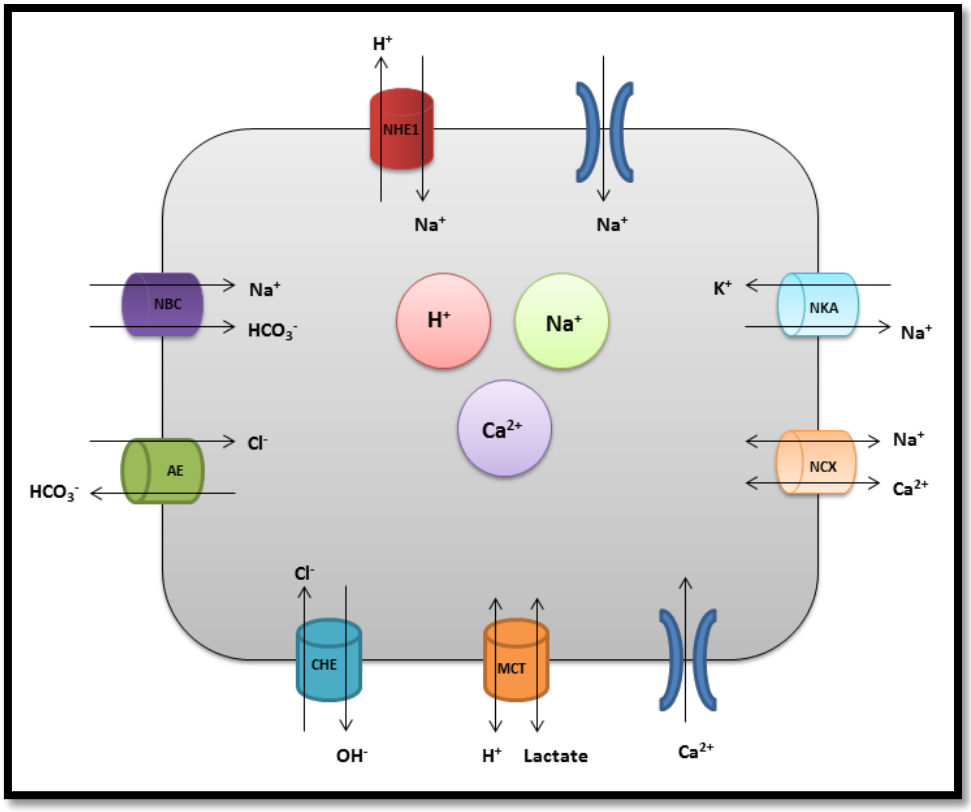


Figure 1.3 Schematic representation of transporters, co-transporters and channels that maintain the ionic homeostasis in ventricular cardiomyocytes. Na^+ influx is controlled by NCX and NHE; Na^+ efflux is controlled by NCX and NKA. H^+ efflux is mediated by MCT, NHE and NBC. H^+ influx occurs through MCT, CHE and AE; Ca^{2+} influx is mediated by Ca^{2+} channels and NCX; Ca^{2+} efflux is mediated through NCX. NHE1, Na^+/H^+ exchanger isoform 1; NCX, Na^+/Ca^{2+} exchanger; NKA, Na^+/K^+ ATPase; NBC, Na^+/HCO_3^- cotransporter; MCT, monocarboxylate transporters; AE, anion exchanger; CHE, Cl^-/OH^- exchanger. A version of this figure can be found in (173).

1.5.2 NHE Isoforms and Distribution

Ten isoforms (NHE 1-10) have been identified. NHE isoforms are composed of 12 transmembrane segments, an N-terminal and C-terminal domains. Each isoform has its own tissue specificity, localization, functions, and inhibitors (174). The NHE proteins can be localized on the plasma membranes as well as in the intracellular compartments. NHE 1-5 are primarily localized on the plasma membranes. The NHE1 is the primary isoform found in the heart whereas NHE2 and NHE3 are found in the stomach, colon and small intestine (9, 175-177). NHE4 is mostly found in the stomach, small intestine, the colon and functions similar to NHE (9). NHE5 is thought to maintain the acidity of synaptic vesicles. It is primarily expressed in non-epithelial tissue such as the brain (9). NHE6-9 are mostly distributed within the intracellular portions of the cell where they function to maintain the intracellular pH and ion concentration (178). NHE6 is primarily found in the heart, brain and skeletal muscles. NHE7, functions slightly different from other isoform as it is capable of exchanging a sodium or a potassium in exchange for protons. It is mainly found in the trans-Golgi network (9). NHE8 can be found in skeletal muscles and kidneys whereas NHE 9 can be found in the endosomes (178). NHE 10, most recently discovered, is found mainly in osteoclasts and function in osteoclast differentiation and survival (179). In our study, we will be focusing on NHE1, the only cardiac specific isoform of NHEs.

1.5.3 Cardiac Specific NHE Isoform 1

NHE1 is the most extensively studied amongst all NHE isoforms and is the only cardiac specific isoform (180, 181). It is located on the plasma membrane and is composed of a twelve transmembrane regions. It functions to remove one intracellular proton in exchange for one extracellular sodium thereby protecting the cell against acidosis (175). The activity of the exchanger is low under physiological conditions. Upon increase in the concentration of protons within the cell, the activity rises sharply to maintain the intracellular pH of the cell (182).

1.5.3.1 Physiological Role of NHE1

NHE1 is vital for many physiological processes (reviewed in (174)). NHE1 plays an important role in regulating the pH and volume of the cells. Apart from maintaining the pH of the cell, NHE1 can also increase the sodium ion concentration within the cell. This increase in intracellular pH activates the AE which results in the increase in chloride ions within the cell. This increase in sodium and chloride ions causes influx of water resulting in cell swelling (183). NHE1 is also implicated to regulate cell growth, cell volume, differentiation, progression, and apoptosis (184-186). Apoptosis causes decrease in cell size and drop in intracellular pH. These effects are counteracted by NHE1. However, its role in apoptosis is dependent upon the cell type. In both human leukemic cell lines and renal proximal tubule, NHE1 seems to protect the cells from apoptosis. In two cytokine-dependent cell lines, NHE1 causes an increase in intracellular pH that results in apoptosis

(187, 188). Moreover, the involvement of NHE1 in apoptosis was demonstrated recently in adult rat ventricular myocytes. This study demonstrated that hypoxia/reperfusion induced phosphorylation of NHE1 was decreased upon silencing of p90 ribosomal S6 kinase1 which decreased apoptosis (189). Moreover the inhibition of NHE1 with amiloride blocked hypoxia/reperfusion induced apoptosis as well (189). There are still significant voids in understanding the role of NHE1 in apoptosis. NHE1 has also been shown to play an important role in the maintenance of cytoskeletal structure, focal adhesion and cell migration (190, 191) (187, 188). More research is needed to understand the role of NHE1 in apoptosis.

1.5.3.2 Regulation of NHE1 Activity in the Myocardium

Regulation of NHE1 mainly occurs through decrease in intracellular pH (192). Apart from acidosis, receptor dependent (phosphorylation/dephosphorylation) and receptor independent pathways (cofactors or protein-protein interactions) also mediate NHE1 activity.

Receptor Dependent Regulation of NHE1

The activity of NHE1 can be stimulated by the activation of a sub type of the G protein coupled receptors (GPCRs). The GPCRs is a family of receptors that are composed of seven transmembrane helices with an extracellular ligand binding domain and an intracellular signal transduction domain (193). The GPCRs constitute four sub types namely, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{11/12}$. The NHE1 activity can be stimulated by the activation

of $G\alpha_q$ via various agonists. (reviewed in (194)). $G\alpha_q$ can be activated by many hormones and paracrine/autocrine stimuli. Moreover, binding of agonist like Phenylephrine (PE) to α_1- adrenoreceptors (ARs), Ang II to Ang II type I receptor type (195), and ET-1 to ET-1 receptor (196) can also activate $G\alpha_q$ signaling thus activating downstream signaling cascade that are translated into cardiovascular responses (Figure 1.4). Binding of these agonist to their receptors raises the intracellular pH threshold for NHE1 activation. Regulation of NHE1, mediated by α_1-ARs, is the most characterized in the myocardium (197). Apart from GPCRs, NHE1 can also be stimulated by thrombin, aldosterone, and lysophosphatidic acid (LPA) (198-200).

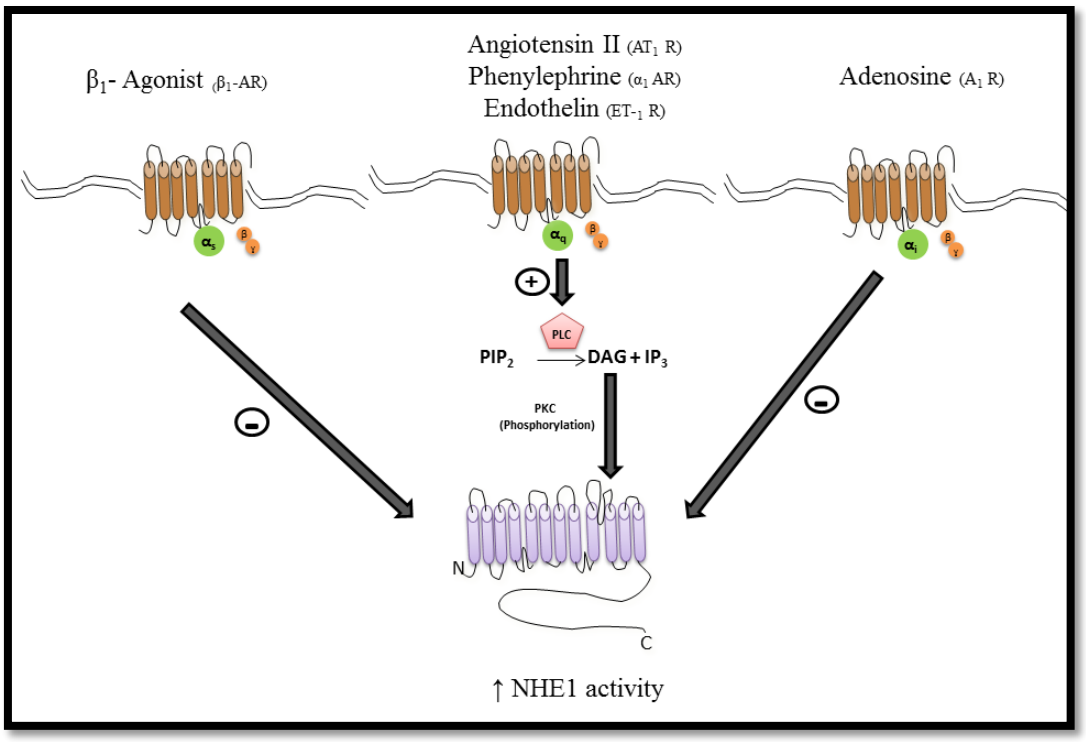


Figure 1.4 G protein coupled receptor regulation of NHE1 in the myocardium. NHE1 activity is inhibited (-) by activation of $G\alpha_s$ and $G\alpha_i$ coupled receptors (β_1-AR, A_1 R, respectively), upon stimulation with corresponding agonists (β_1-agonists and adenosine, respectively).NHE1 activity is stimulated (+) by the activation of $G\alpha_q$ coupled receptors (AT_1R, α_1-AR. ET_1) upon activation with corresponding agonists (phenylephrine, endothelin-1 and angiotensin II). NHE1; sodium proton exchanger isoform 1; AT_1R, angiotensin II receptor type 1; α_1- AR., α_1-adrenoreceptors; β_1-AR, β_1- adrenoreceptors; A_1 R, adenosine receptors. A version of this figure can be found in (173)

1.5.3.3 Role of NHE1 in Cardiovascular Diseases

NHE1 in Hypertension

It has been shown that the activity of the NHE1 is increased in essential hypertensive patients (201). It has been suggested that hypertension can occur as a result of over activation of the NHE1. Over activation of NHE1 can cause the accumulation of sodium ions inside the cell. This activates the NCX in reverse mode which expels sodium ions outside in exchange for calcium ions, increasing the calcium ion concentration inside the cell (202). A study done on rats with hypertension and pathological CH showed that the inhibition of NHE1 was able to decrease systolic blood pressure, normalize NHE1 activity, and regress CH (203). Despite various studies investigating the role of NHE1 in hypertension, its role is still not clear. More studies are needed to clearly define its involvement in hypertension.

NHE1 in Cardiac Hypertrophy

Results from multiple *in vivo* models of CH have reported an increase in NHE1 mRNA expression, protein expression or activity (204, 205). Interestingly, the increase in NHE1 activity can occur independent of the increase in protein expression (206). Moreover, CH was attenuated upon pharmacological inhibition of NHE1 in multiple *in vivo* models (179). CH was abrogated in rabbits subjected to volume and pressure over-load upon NHE1 inhibition which prevented further development of heart failure (207). In MI models, inhibition of NHE1 was demonstrated to decrease right and left ventricular hypertrophy

(208-210). Additionally, transgenic mice that were over expressed with active human NHE1 have provided further evidence of NHE1 mediated CH, contractile dysfunction and heart failure (54).

1.5.3.4 NHE1 Inhibitors

Many NHE1 inhibitors have been developed over the years. They have been developed further in attempts to increase their selectivity to the various isoform of NHE1. The first inhibitor to be developed were amiloride derivatives like 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and 5-(N, N-Dimethyl) amiloride hydrochloride (DMA) (211). A second class of inhibitors were developed with increased selectivity for NHE1. This class included N-(2-methyl-4,5-bis(methylsulfonyl)-benzoyl)-guanidine hydrochloride) (EMD87850), cariporide (HOE type), eniporide (EMD85131) (212).

To date, the EMD and HOE type inhibitors have proven to be the most suitable for i*n vitro* and *in vivo* inhibition of NHE1 (209, 213).

1.6 The Cross Talk Between Cat B, MMP-9 and NHE1

Progression of the heart to failure is primarily caused due to significant remodeling of both the ECM and subcellular organelles which occurs as a result of the activation and increased proteolytic activities of proteases such as Cat B and MMP-9 (77, 214). Previous studies have suggested that the activation of Cat B is induced by the acidification of the peri and extracellular space (86, 132, 133). In various forms of carcinomas, this pericellular acidification coincides with the activation of NHE1 (132, 174). Increased

activation of NHE1, similar to Cat B, is involved in the pathogenesis of various cardiac diseases including CH (50, 96, 215, 216). Moreover, the activation NHE1 has been shown to activate Cat B in various reports. CD44 was shown to interact with NHE1 which created an acidic microenvironments leading to Cat B activation in a breast cancer model (132). Moreover, NHE1 and Cat B have shown to directly interact with each other and cause ECM degradation in another breast cancer model (133). Taken together, the evidence suggests that NHE1, through its pH regulating property, might be mediating the activity of Cat B in pathological states.

A previous report has demonstrated that pericellular acidification redistributed the Cat B containing lysosomes to the cell surface and caused the secretion of Cat B into the extracellular compartment (86). Interestingly, the NHEs have also shown to cause acidic extracellular pH which induced lysosome trafficking and subsequent release of Cat B into the ECM in a prostate cancer cells (136). Moreover, several broad and specific NHE inhibitors were able to inhibit this effect, i.e. redistribution of lysosomes and subsequent secretion of Cat B (136). Once into the extracellular compartment, Cat B can degrade the ECM (68) and facilitate further ECM degradation by activating other proteases such as MMP-9 (217, 218). MMP-9 activity has been shown to be increased in various models of heart failure (43, 219) (220, 221). Previous studies have also shown that MMP-9 activity was increased in CCL39 cells upon the stimulation of NHE1 with phenylephrine (222). Interestingly, Cat B and MMP-9 were shown to directly interact with NHE1 and cause ECM degradation in breast cancer (133). Whether NHE1 induces the activation of Cat B,

which in turn activates MMP-9 and contributes to cardiomyocyte hypertrophy remains unclear.

1.7 Thesis Objectives

CH is one of the leading causes of death worldwide. Despite advances in cardiovascular research, therapeutic targets that regress CH still remain undiscovered. Hence, there is an immediate need to further investigate the signaling pathways that mediate CH in order to identify potential therapeutic targets. One of the hallmarks of CH is ECM remodeling. Multiple studies have shown an important role of proteases, specifically Cat B and MMP-9, in the degradation of the ECM. Cat B is activated under acidic conditions, such as in conditions where there is an overexpression of the cardiac specific pH regulator, NHE1. Various studies have shown that enhanced expression and activity of the NHE1 contributes towards CH. Moreover, Cat B has also been shown to induce apoptosis and autophagy in conditions of heart failure. Increased autophagy can result in the release of Cat B into the ECM where it can activate MMP-9 which can result in further ECM degradation. Whether there is a link between NHE1 and Cat B-MMP-9 under pH changes in the CH signaling pathway remains unknown. The purpose of this study was to (a) Investigate the role of Cat B in the NHE1 induced cardiomyocyte hypertrophic response, (b) Investigate the signaling pathways that cause the Cat B mediated hypertrophic response, (c) Investigate the role of apoptosis and the autophagy-lysosomal pathway in the NHE1 induced cardiomyocyte hypertrophic response. Our **hypothesis** is that activation of NHE1 induces Cat B which in turn activates MMP-9, which leads to cardiomyocyte hypertrophy. Furthermore, induction

of Cat B also causes the activation of apoptosis and autophagy in the NHE1 induced cardiomyocyte hypertrophic response.

Chapter 2: MATERIAL AND METHODS

2.1 Materials

All chemicals were purchased from BD Biosciences (San Jose, CA), Fisher Scientific (Ottawa, ON) or Sigma (St. Louis, MO). The specific NHE1 inhibitor, N-(4,5-Bis-methansulfony-2-methylbenzoyl) guanidine (EMD 87580/EMD), which was made by Harlan Laboratories (Madison, WI) was a generous gift of Dr. N. Beier of Merck KGaA (Frankfurt, Germany). The specific Cat B inhibitor, CA-074 methyl ester (CA-074Me) was purchased from Santa Cruz Biotechnologies (sc-214647). CA-074Me is a cell permeable methyl ester of CA-074. In our study, we aimed to investigate the effect of intracellular Cat B inhibition and thus used CA-074Me instead of CA-074 (223). Primary antibodies used for Western blotting including rabbit polyclonal Cat B (sc-13985), rabbit polyclonal MMP-9 (sc-10737) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-NHE1 (ab67314), anti- OPN (ab8448), anti-α-tubulin (ab4074), and anti-goat (ab97051) antibodies were from Abcam (Cambridge, MA). Rabbit polyclonal anti-LC3 A/B (12741) and rabbit monoclonal anti-caspase-3 (9665) were purchased from Cell Signaling (Table 2.1).

Table 2.1 Primary and secondary antibodies used for Immunoblotting. Primary and secondary antibodies were used for the analysis of protein expression of Cat B, NHE1, MMP-9, OPN, caspase-3 and LC-3 A/B. Cat B, cathepsin B; NHE1, Na^+/H^+ exchanger isoform-1; MMP-9, matrix metalloproteinase-9; OPN, osteopontin; LC-3 A/B, microtubule associated light chain 3

	Primary Antibody	**Company and Catalog Number**	**Band recognition sites**
Cat B	Rabbit polyclonal anti-Cat B	Santa Cruz Biotechnology (Santa Cruz, CA); sc-13985	37 (pro Cat B) and 25 (active Cat B) kDa
MMP-9	Rabbit polyclonal anti-MMP-9	Santa Cruz Biotechnology (Santa Cruz, CA); sc-10737	92 kDa
NHE1	Rabbit polyclonal anti-NHE1	Abcam (Cambridge, MA); ab67314	91 kDa
α-tubulin	Rabbit polyclonal anti-α-tubulin	Abcam (Cambridge, MA); ab4074	50 kDa
LC3 A/B	Rabbit monoclonal anti-LC3 A/B	Cell Signaling; 12741	14 (LC3-II) and 16 (LC3-I) kDa
Caspase-3	Rabbit monoclonal anti-caspase-3	Cell Signaling; 9665	17, 19 and 35 kDa
OPN	Rabbit polyclonal osteopontin	Abcam; ab8448	66 and 32 kDa
Rabbit anti goat	Rabbit polyclonal anti-goat	Abcam; ab97051	-

2. 2 *In Vitro* Studies Using the Rat Embryonic Myoblast Cell Line H9c2 (A summary of the experimental model can found in figure 2.3)

H9c2 myoblasts, a clonal cell line derived from the embryonic BD1X rat heart tissue (224) were obtained from European Collections of Cell Cultures (ECACC) and cultured in DMEM/F12 1:1 culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere (95% O_2-5% CO_2) (224). The H9c2 cell line shows similar response to hypertrophic parameters as the primary cardiomyoblasts and hence can be used as a model for *in vitro* study of CH (225). The H9c2 cell line was originally derived from embryonic rat ventricular tissue, which is important as cardiac hypertrophy mainly occurs in the ventricular muscle of the heart (226). The well-established Ang II hypertrophic model was used for inducing cardiomyocyte hypertrophy and stimulating NHE1 (227). Previous studies have demonstrated that exogenous administration of prohypertrophic agents such as Ang II, stimulates NHE-1 activity (228, 229).

Treatment Workflow

Upon becoming confluent, cells were seeded at a density of 2.0×10^6 cells per 35mm culture dishes and cultured for 48 hours. After 24 hours, the media was changed to serum-free maintenance medium for a further 24 hours.

a. ***General treatment plan:*** The cells were then treated with Ang II (10 µM) (refer section 3.1.3 for use of the specific concentration), Ang II+EMD, Ang II+CA-074Me, EMD (10 µM) (51), or CA-074Me (10 µM) (216) alone for 24 hours (refer

section 3.1.3 for use of the specific time point) (Figure 2.1). Ang II and EMD were dissolved in distilled water and CA-074Me was prepared in dimethyl sulfoxide (DMSO). The mentioned vehicle, concentration and time point did not have any significant effect on the cell viability (Figure 2.2) which was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

b. ***Treatment plan for the determination of Ang II concentration to be used:*** The cells were then treated with 1, 10, or 100 µM or 1, 10, or 100 nM Ang II for 24 hours (refer to section 3.1.3).

c. ***Treatment plan for the determination of Ang II time point to be used:*** The cells were then treated with 10 µM Ang II for 0, 3, 6, 12 and 24 hours (refer to section 3.1.3).

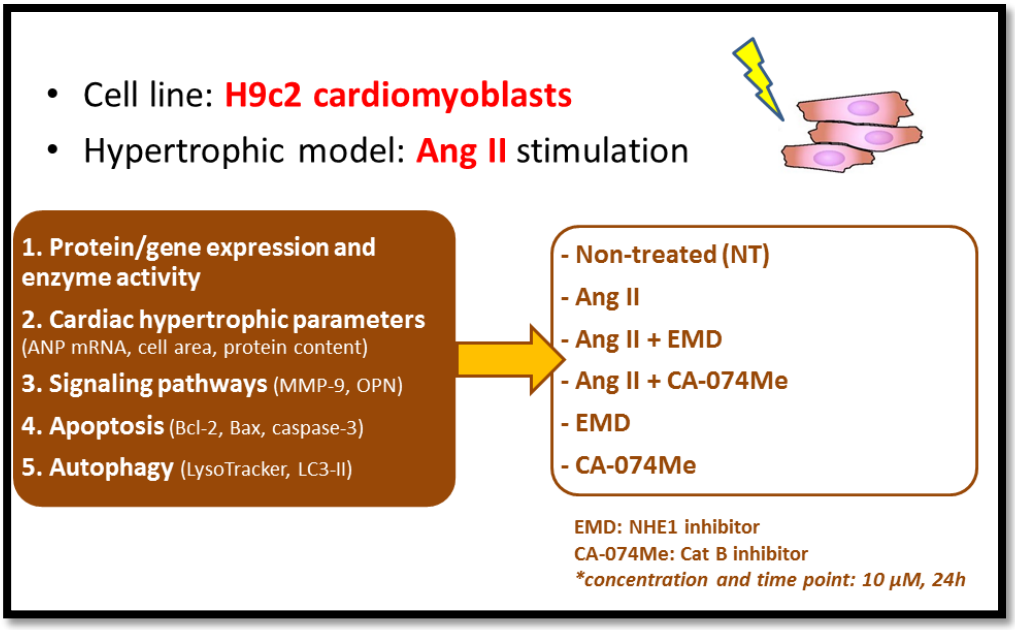


Figure 2.1 Schematic representation of treatment groups and parameters analysed. For analysis of protein/gene expression, CH markers, signaling pathways (MMP-9, OPN), apoptosis and autophagy the H9c2 cardiomyoblasts were treated with Ang II (10 µM), Ang II+EMD, Ang II+CA-074Me, EMD (10 µM), or CA-074Me (10 µM) alone for 24 hours. CH, cardiac hypertrophy; MMP-9, matrix metalloproteinase-9; Ang II, angiotensin II.

MTT Assay

Cell viability was measured using MTT assay, based on the MTT conversion into formazan crystals using mitochondrial dehydrogenases. Briefly, H9c2 cells were plated at a density of 1 x 10^4 cells/well in 96-well plates. After different treatment for 24 hours, the culture medium was replaced with 200 µL of MTT solution (5 mg/mL stock solution in PBS, diluted with culture medium to the final concentration 0.5 mg/mL). After 4 hours of incubation at 37^0 C, this solution was removed and the produced formazan was solubilized in 150 µL of DMSO. The absorbance was measured at 550 nm using an automated

microplate reader. The results confirmed that the mentioned concentrations and the vehicle did not have any significant effect on the cell viability (Figure 2.2).

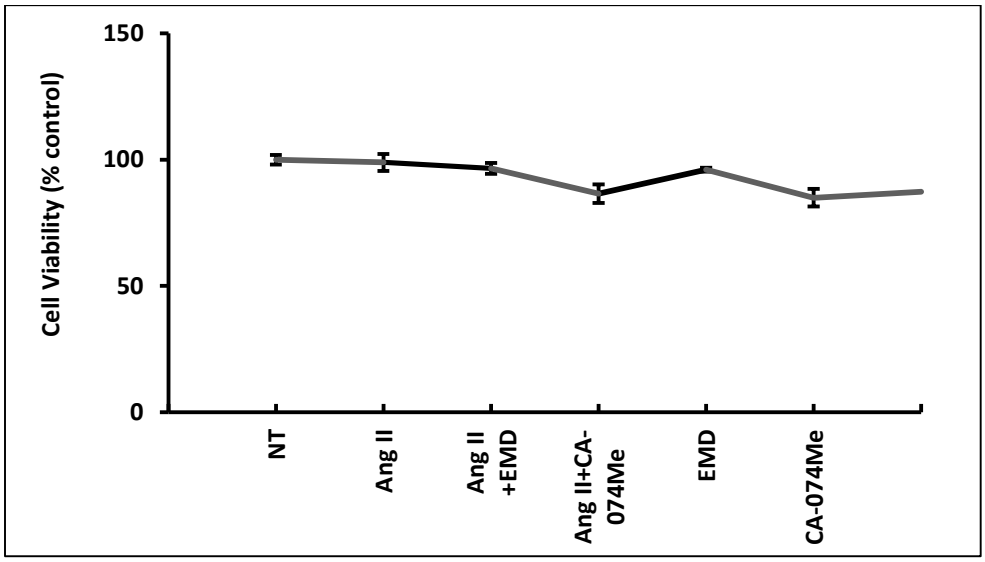


Figure 2.2 Effect of different treatment groups on cell viability. The cell viability was tested 24 hours after treatment of H9c2 cardiomyocytes with or without EMD or CA-074Me by measuring the conversion of MTT to formazan crystals. Data is expressed as % of control (non-treated) ±SEM (n=3).

2.2.1 Immunoblotting

2.2.1.1 Protein Expression in Cell Lysates

Following treatment, H9c2 cardiomyoblasts were lysed using radio-immunoprecipitation protein assay (RIPA) buffer as described earlier (55). Briefly, cell lysates were centrifuged at 2340 g for 15 minutes at 4°C and the supernatant containing the proteins were collected.

2.2.1.2 Protein Expression in the Culture Media

Following treatment, the media was collected and centrifuged at 37 g for 5 minutes at 4°C. The supernatant was collected and stored at -20°C for

concentration. The media was concentrated using a vacuum concentrator, Concentrator plus (Eppendorf, Germany) at room temperature for approximately 8 hours.

Total amount of protein present was quantified using the DC protein assay kit (Biorad) and equal amounts of protein were loaded on the gels. 20-40 µg of proteins were resolved on 15% or 9% SDS-PAGE. The membranes were probed with anti-Cat B antibody, rabbit polyclonal anti-MMP-9, rabbit polyclonal anti-NHE1, rabbit monoclonal anti-LC3 A/B, rabbit monoclonal caspase-3, and rabbit polyclonal OPN (refer table 2.1). Anti-α-tubulin was used as a loading control. Immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham Biosciences) and imaged using the Alpha Innotech FluorChem Imager (Protein Simple, California, USA).

2.2.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to analyze Cat B and ANP mRNA expression. RNA was extracted using the Trizol RNA extraction protocol. 2 µg of total RNA was reverse transcribed into cDNA using High capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol (Table 2.2). 50-175 ng from the cDNA product was used for each PCR reaction. Following an initial denaturation of 3 minutes at 94°C, the samples were denatured at 94°C for 45 seconds, annealed at 60°C for 30 seconds and extended at 72°C for 1 minute. After 35 cycles of denaturing, annealing and extending, the reaction was terminated at 72°C for 10 minutes for final extension (Table 2.3). Cat B cDNA, ANP cDNA, Bcl-2 cDNA and Bax cDNA were amplified

separately using the below mentioned primer sequences (Table 2.4). The results were normalized using the control β-actin. β-actin is a housekeeping gene and is hence used to normalization for sample-specific differences (230). PCR products were then electrophoresed on 2% agarose gels stained with ethidium bromide. The mRNA bands were imaged using the Alpha Innotech FluorChem Imager (Protein Simple, California, USA). The changes in the mRNA levels were normalized to β-actin and quantified using Scion Image software (Scion Corporation, USA).

Table 2.2 Reverse transcription conditions used for converting RNA to cDNA. RT conditions used for the analysis of Cat B and ANP, Bcl-2, Bax mRNA levels. β-actin was used as an internal reference gene. RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; Cat B, cathepsin B; ANP, atrial natriuretic peptide; mRNA, messenger ribonucleic acid; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein.

Step	**Temperature (^{0}C)**	**Time**
1	25	10 min
2	37	2 hours
3	85	5 min
4	4	indefinitely
Lid Temperature	95	

Table 2.3 Semi-quantitative Reverse Transcription-PCR conditions (RT-PCR). RT-PCR for the analysis of Cat B and ANP mRNA levels. β-actin was used as an internal reference gene.

Step	**Temperature (^{0}C)**	**Time**	
Initial Denaturation	94	3 min	
Denaturation	94	45 sec	35 cycles
Annealing	60	30 sec	
Extension	72	1 min	
Final Extension	72	10 min	
Hold	4	indefinitely	
	End		

2.2.3 Real-Time Polymerase Chain Reaction

Real-Time PCR was used to analyze Bcl-2 and Bax mRNA expression. RNA was extracted using the Trizol RNA extraction protocol. 2 µg of total RNA was reverse transcribed into cDNA using High capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol (Table 2.2). 2 µL from the cDNA product was used for each Real-Time PCR reaction. Fast Sybr Green Master Mix (Applied Biosystems) was used for Real-Time PCR. Reaction mixture contained 10 µL Sybr Green, 2 µLof cDNA, 0.5 µL of 20 µM forward and reverse primers (Table 2.4) in a total volume of 20 µL. The microtiter plate was then centrifuged at 900 rpm for 5 to 10 seconds. 7500 fast Real-Time PCR System (Applied Biosystems) was used for the Real-Time PCR run. The holding stage conditions were 95^{0}C for 20 seconds. Thermal cycling conditions were

40 cycles of 3 seconds at 95^0C and 30 seconds at 60^0C. The melt curve stage step 1 conditions were 15 seconds at 95^0C, followed by 60 seconds at 60^0C and finally 15 seconds at 95^0C. The results were normalized using the control β-actin. β-actin is a housekeeping gene and is hence used to normalization for sample-specific differences (230). The gene expression of Bcl-2 and Bax was performed in triplicates.

Table 2.4 Synthetic oligonucleotide primer sequences for Semi-quantitative Reverse Transcription-PCR. Primer sequences of Cat B, ANP, Bcl-2, Bax mRNA levels. β-actin was used as an internal reference gene.

Gene	Direction	Primer Sequence
β-actin	Sense	5′-CGT CAT CCA TGG CGA ACT GG-3′
	Antisense	5′-ACG CAG CTC AGT AAC AGT CC-3′
CTSB	Sense	5′-GGG GGA AAT CTA CAA AAA TG-3′
	Antisense	5′-AAA GAC TCC TAT CTG CCT CAC T-3′
ANP	Sense	5′-CTG CTA GAC CAC CTG GAG GA-3′
	Antisense	5′-AAG CTG TTG CAG CCT AGT CC-3′
Bcl-2	Sense	5′-AGC GTC AAC AGG GAG ATG TGA-3′
	Antisense	5′-GAT GCC GGT TCA GGT ACT CA-3′
Bax	Sense	5′-CCA AGA AGC TGA GCG AGT GTC TC-3′
	Antisense	5′-AGT TGC CAT CAG CAA ACA TGT CA-3′

2.2.4 Measurement of Cardiac Hypertrophic Markers

In order to investigate the role of NHE1 in the cardiomyocyte hypertrophic cascade we looked at three cardiomyocyte hypertrophic markers namely, ANP mRNA, protein content and cell area. Increase in ANP mRNA and protein synthesis are one of the hallmarks of CH and heart failure (231). Additionally, it was shown that treatment with hypertrophic agonists like ET-1 and Ang II induced an increase in cell surface area (232, 233). Thus, their levels were considered as good markers of cardiomyocyte hypertrophy.

2.2.4.1 Measurement of Cell Surface Area of H9c2 Cardiomyoblasts

H9c2 cardiomyoblasts were plated at an average density of $3x10^4$ per 35 mm dish, which allowed for clear distinction of cells in DMEM F12 media containing the 10% FBS and 1% penicillin/streptomycin and cultured for 24 hours. Upon becoming confluent after 24 hours, the media was changed to serum-free maintenance medium for a further 24 hours and the cells were then treated with Ang II (10 µM), Ang II+EMD (NHE1 inhibitor), Ang II+CA-074 Me (Cat B inhibitor), EMD (10 µM), or CA-074Me (10 µM) alone for 24 hours by directly adding them to the tissue culture medium. Following treatment, the media was aspirated and the cells were washed twice with 1X Phosphate Buffer Saline (PBS). The cells were then fixed with 4% formaldehyde and stained with 0.5% crystal violet in 2% ethanol. At least 10 cells were averaged from three dishes and represented as one n value. The cells were visualized on a bright field through Carl Zeiss AxioCam microscope (Carl Zeiss, Germany). Cell area was measured from digitized images using the AxioVision Imaging software (Carl Zeiss Microimaging, New York, NY).

2.2.4.2 Measurement of Protein Content of H9c2 Cardiomyoblasts

Protein content was measured as described previously (234). Briefly, the cardiomyoblasts were washed twice in 1X PBS and collected by trypsinization. The total number of cardiomyoblasts was calculated using a hemocytometer. Protein concentration of cardiomyoblasts lysed in RIPA buffer was measured using the DC protein assay kit (Biorad). Protein content was determined by dividing the total amount of protein (µg) by the total number of cardiomyoblasts. Results were expressed as % of control (non-treated).

2.2.5 Measurement of Cathepsin B Enzyme Activity

Activity of cathepsin B was determined with cathepsin B activity fluorometric assay kit according to the manufacturer's protocol (abcam) and as previously described (235). Briefly, the cells were lysed in 50 µL kit lysis buffer. Cell lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant containing the proteins were collected. Total amount of protein present in each sample was quantified using the DC protein assay kit from Biorad according to the manufacturer's instructions. 50 µg of proteins were used for cathepsin B activity assay. Proteins were incubated at 37°C for 1–2 hours 10 mM Ac-RR-AFC (substrate for cathepsin B). After incubation for 2 hour, fluorescence was measured with a fluorescence microplate reader (SpectraMax Geminis XS; Molecular Devices, Inc.) at 400/505nm (excitation/emission; for cathepsin B activity).

2.2.6 Measurement of NHE1 Activity

Cardiomyoblasts plated on coverslips were loaded with 3 µg/mL pH sensitive dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM). The change in H^+ concentration was measured using a PTI Deltascan spectrofluorometer (Photon Technology International, Doha, Qatar). The excitation wavelengths were set at 502.5 nm and 440 nm and the emission wavelength was set at 528.7 nm. The coverslip was initially maintained in a pre-warmed solution of Na^+-normal buffer (mM (135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$, 5.5 Glucose, 10 HEPES) at 37°C and was then pulsed with 50 mM ammonium chloride to induce an acid load. Following acidification, coverslips were placed

in a 37°C pre-warmed solution of Na^+-free buffer (135mM N-methyl-D-glucamine, 5mM KCl, 1.8mM CaCl2, 1mM MgSO4, 5.5mM Glucose, 10mM HEPES) that was pre-warmed to 37°C and adjusted to a pH of 7.3 until a steady acidic pH was reached. The coverslip was then placed in Na^+-normal buffer to allow the cardiomyoblasts to recover (176). Each coverslip was equilibrated in a three-step pH calibration buffer solution containing 135 mM N-methyl-glucamine and KCl, 10 µM nigericin and adjusted to a pH of 8, 7 or 6 (236). The three-step pH calibration was used to generate a standard curve in which the fluorescence output measurements were converted into intracellular pH. The initial rate of recovery following an induced acid load was measured and used as an indicator of the NHE1 activity.

2.2.7 Measurement of MMP-9 Gelatinolytic Activity by Gelatin Zymography in the Conditioned Media

SDS-substrate (gelatin) gels were prepared by adding gelatin (final concentration of 0.2%) to a 7.5% standard Laemmli acrylamide polymerization mixture. 80 µg of protein from concentrated media samples was loaded per lane without reduction. Following electrophoresis, gels were washed thrice for 20 minutes each with 2.5% Triton X-I00. Gels were then rinsed and incubated for 24 hours at 37°C in substrate buffer (50 mM Tris HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3, pH 7.6). After incubation, gels were stained for 1 hour in 1% coomassie blue R-250 in acetic acid:methanol:water (1:2.5:6.5), destained in the same solvent and scanned for gelatinolytic activity using BioRad gel doc imaging

system (Bio-Rad Inc., California, USA). The bands were quantified using Scion Image software (Scion Corporation, USA). Bands for MMP-9 were visualized at 92 kDa.

2.2.8 Intracellular Localization of Lysosomes Using LysoTracker Red Dye

Following treatment, the H9c2 cardiomyoblasts were washed with PBS and incubated in DMEM medium containing 75 µM LysoTracker Red DND-99 (Invitrogen), a cell-permeable acidotropic probe that selectively labels vacuoles with low internal pH, for 30 minutes. The media was removed and the cells were incubated with 1 µM HOECST for 30 minutes. The cells were then evaluated under a fluorescence microscope (OlympusIX70, Olympus Corp.).

2.3 *In Vivo* Studies Using of Hearts from Transgenic Mice Overexpressing NHE1 (K-Line); Measurement of Cathepsin B Protein Expression (A summary of the experimental model can found in figure 2.3)

Cat B protein expression in the hearts of transgenic mice overexpressing NHE1 (53) was investigated by immunohistochemical analysis. All experimental procedures involving mice were in accordance with institutional guidelines on Animal Experimentation and were under a French Ministry of Agriculture license and the Institutional Animal Care and Use Committee at Qatar University, Doha, Qatar. The Institutional Animal Care and Use Committee at Qatar University, Doha, Qatar has specifically approved this project (Research Ethics Approval Number: QU-IACUC 007/2012). The mice hearts were collected at 13 weeks and were sectioned into 7 µm

sections and dried overnight. The sections were then deparaffinized and rehydrated with xylene and alcohol, respectively. The antigens were retrieved with Ventana CC1 buffer (Tris/EDTA buffer, pH 8) for 4 minutes at 95°C and then for 12 minutes at 100°C. The endogenous peroxidase was quenched with 3% H_2O_2 in PBS for 30 minutes. Heart sections were then blocked with Ab Diluent (ROCHE) for at least 20-30 minutes in a moist chamber. The sections were then probed with primary antibodies to Cat B at a dilution of 1:50 in Ab Diluent (ROCHE) for 1 hour at 37^0C. Biotinylated anti rabbit IgG secondary antibody (RPN1004V,GE Healthcare) was then added at a dilution of 1:100 in Ab Diluent (ROCHE) for 1 hour at 37°C. Finally, the 3,3-diaminobenzidine (DAB) kit was used. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired on NIKON microphot-FXA.

2.4 Statistical Analysis

All values expressed are compared to control (non-treated) ± SEM. Since the data represented values with unequal variances that followed a normal distribution, the upaired student's t tests was used to compute differences between groups. A p value of less than 0.05 was considered a significant difference. * represents $p < 0.05$ vs. control (non-treated) and # represents $p < 0.05$ vs. Ang II.

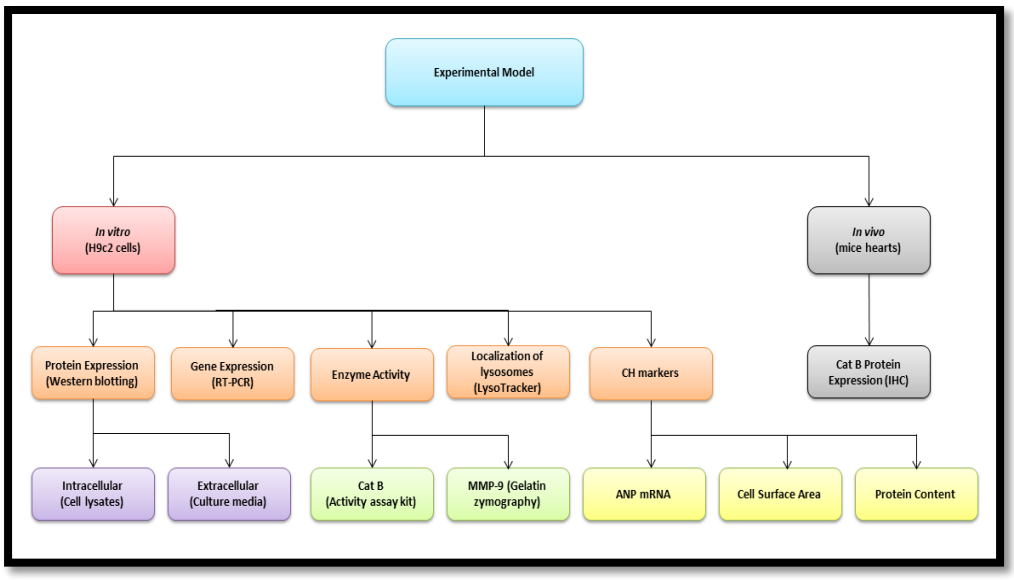


Figure 2.3 Experimental model used to determine the role of Cat B in NHE1-induced cardiomyocyte hypertrophic response. Cardiomyocytes were investigated for Cat B protein (*in vitro* and *in vivo*) and gene expression. Cardiomyocytes were also characterized for parameters of cardiac hypertrophy including ANP mRNA levels, cell surface area, and protein content. Hypertrophic signaling pathways were also examined to determine their involvement in the NHE1-induced cardiomyocyte hypertrophic response. RT-PCR, reverse transcription-polymerase chain reaction; CH, cardiac hypertrophy; Cat B, cathepsin B; MMP-9, matrix metalloproteinase-9; ANP, atrial natriuretic peptide; IHC, immunohistochemistry.

Chapter 3: RESEARCH FINDING

3.1 *In vitro* Studies Using the Rat Embryonic Myoblast Cell Line H9c2 to Investigate the Cellular Interplay between NHE1 and Cat B

3.1.1 Measurement of NHE1 Activity Following Stimulation with Ang II

Previous reports have demonstrated a simultaneous increase in NHE1 activity upon stimulation with Ang II in cardiomyocytes (229). In order to determine whether Ang II induces NHE1 activity in H9c2 cardiomyocytes, the cells were treated with 100 nM Ang II for 18 hours, with or without EMD. NHE1 activity assay revealed that NHE1 activity was significantly elevated with 100 nM Ang II concentration at the 18 hour time point, which was significantly regressed by EMD (Figure 3.1).

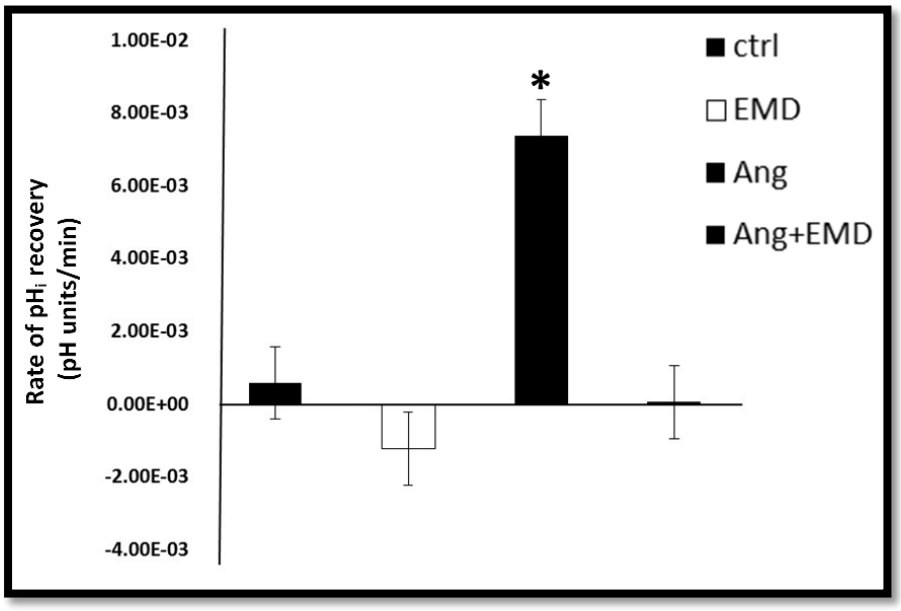


Figure 3.1 Treatment with Ang II induces an increase in NHE1 activity in H9c2 cardiomyoblasts. Quantification of NHE1 activity in cell lysates of H9c2 cells treated with 100 nM Ang II, with or without EMD for 18 hours (n=4). *$p < 0.05$ vs. control.

3.1.2 Measurement of NHE1 Protein Expression Following Stimulation with Ang II

Previous reports have a demonstrated a simultaneous increase in NHE1 activity upon stimulation with Ang II in cardiomyocytes but not NHE1 protein expression (229). Our previous findings revealed that Ang II induced an increase in NHE1 activity which was blocked by EMD (Figure 3.1) In order to determine whether Ang II induces an increase in NHE1 protein expression in H9c2 cardiomyocytes as well, the cells were treated with 10 µM Ang II for 24 hours, with or without EMD or CA-074Me. Immunoblot analysis revealed that NHE1 protein expression was not changed with 10 µM Ang II concentration at the 24 hour time point (Figure 3.2).

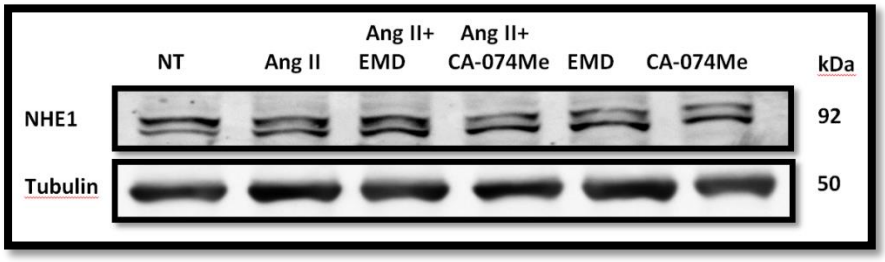


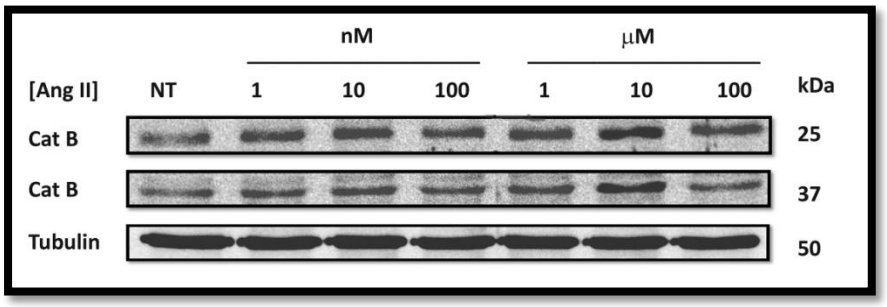
Figure 3.2 Treatment with Ang II causes no change in NHE1 protein expression in H9c2 cardiomyoblasts. Representative Western blot of NHE1 protein expression in cell lysates of H9c2 cells treated with 10 µM Ang II for 24 hours, with or without EMD or CA-074Me (n=1). Immunoblotting was against NHE1 (92 kDa) and α-tubulin (50 kDa).

3.1.3 Determination of the Optimal Concentration and Time Point At Which Ang II Induces Cat B

Previous studies have shown that Cat B is optimally active under acidic pH (86, 237) Similarly, NHE1 is the primary regulator of intracellular pH in cardiomyocytes. Cat B and NHE1 have independently been shown to mediate CH (36, 53, 91, 214). Moreover, the activation NHE1 has been shown to activate Cat B in a breast cancer model (132). In order

to determine whether the activation of NHE1 induces Cat B protein expression in H9c2 cardiomyoblasts, H9c2 cardiomyoblasts were treated with 1, 10, or 100 μM or 1, 10, or 100 nM Ang II for 24 hours. Previous reports and our findings (Figure 3.1) have demonstrated that NHE1 activity increases upon stimulation with Ang II in ventricular myocytes (227), and as such Ang II has been used as experimental model to stimulate NHE1 activity. Immunoblot analysis revealed that Cat B protein expression, appearing at 37 kDa (pro form) and 25 kDa (active form) (238), was significantly elevated following stimulation with 10 μM Ang II (180.22 ± 23.40% Ang II vs. 100% control, 25 kDa; and 180.07 ± 21.97% Ang II vs. 100% control, 37 kDa; $P<0.05$) (Figure 3.3). Our results demonstrate that stimulation of NHE1 with Ang II in H9c2 cardiomyoblasts induces Cat B protein expression.

A.



B.

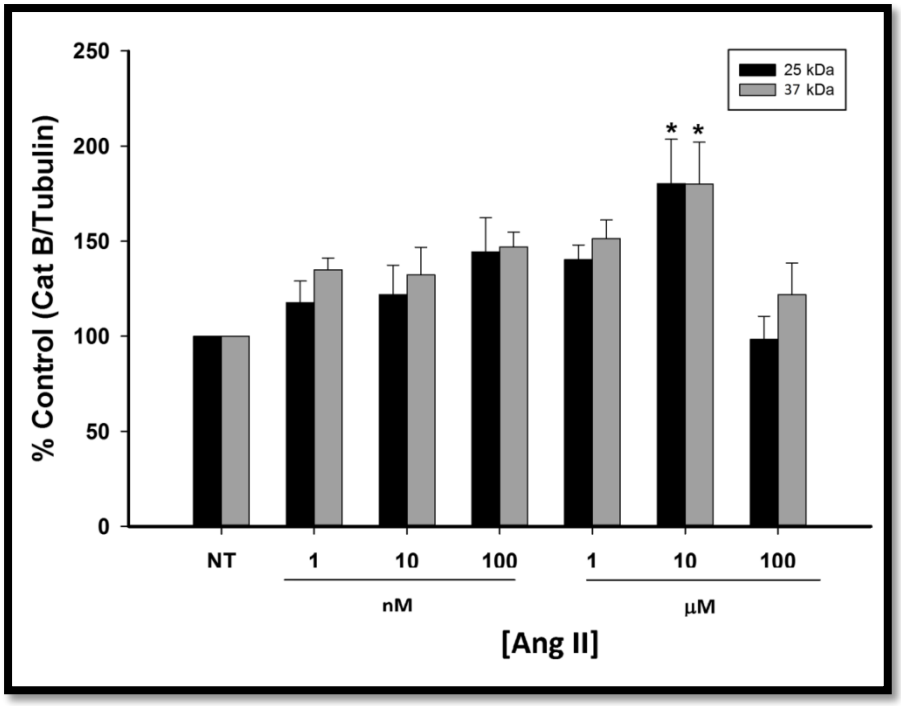


Figure 3.3 Stimulation of Na^+/H^+ exchanger isoform 1 through Ang II elicits a concentration-dependent increase in Cat B protein expression in H9c2 cardiomyoblasts. A. Representative Western blot of Cat B protein expression in cell lysates of H9c2 cells treated with 1, 10, or 100 μM or 1, 10, or 100 nM Ang II for 24 hours (n=7). Immunoblotting was against Cat B (pro form at 37 kDa and active form at 25 kDa) and α-tubulin (50 kDa). **B.** Quantification of relative levels of Cat B protein expression (n=7). Results expressed as % control (α-tubulin) ± %SEM. *$p < 0.05$ vs. control.

In order to determine the time point at which Ang II maximally induces Cat B protein expression in H9c2 cardiomyocytes, the cells were treated with 10 μM Ang II for

0, 3, 6, 12 or 24 hours. Immunoblot analysis revealed that Cat B protein expression, appearing at 37 kDa (pro form) and 25 kDa (active form), was maximally increased at the 24 hour time point with 10 µM Ang II concentration (Figure 3.4). It is to be noted here that we saw a doublet at the 37 kDa band size which was not observed earlier (Figure 3.1). This can be attributed to a longer run time of the Western blot.

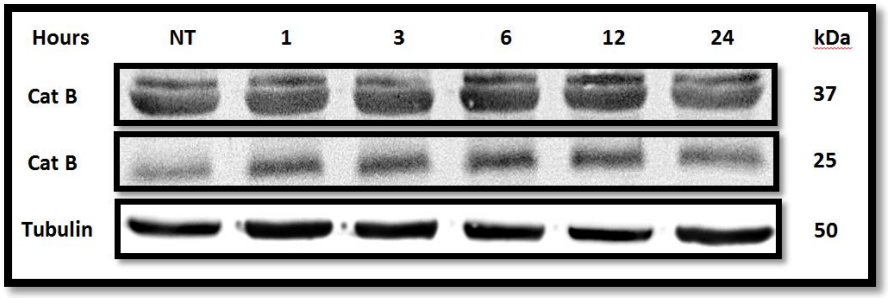


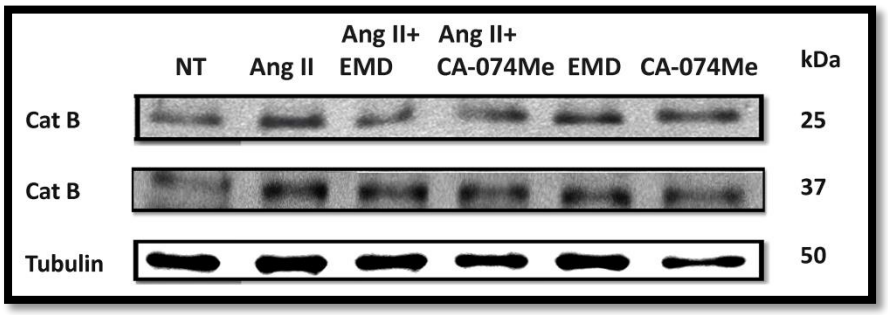
Figure 3.4 Stimulation of Na^+/H^+ exchanger isoform 1 through Ang II elicits a time-dependent increase in Cat B protein expression in H9c2 cardiomyoblasts (representative Western blots). Representative Western blot of Cat B protein expression in cell lysates of H9c2 cells treated with 10 µM Ang II for 0, 3, 6, 12 or 24 hours (n=1). Immunoblotting was against Cat B (pro form at 37 kDa and active form at 25 kDa) and α-tubulin (50 kDa).

3.1.4 Analysis of Cat B in the Intracellular Compartment

3.1.4.1 Measurement of Cat B Protein Expression in Cell Lysates

The activation of NHE1 has been shown to activate Cat B in a breast cancer model (132). In order to confirm whether the Ang II stimulated increase in Cat B protein expression was in part due to the activation of NHE1 and whether this increase could be regressed by NHE1 inhibitor or Cat B inhibitor, the cells were further treated with or without EMD or CA-074Me. Immunoblot analysis revealed that Cat B protein expression, both pro and active forms, was significantly elevated at the 10 µM Ang II concentration (136.56 ± 9.4% Ang II vs. 100% control, 37 kDa and 169.84 ± 14.24% Ang II vs. 100% control, 25 kDa; $P<0.05$) and this increase was regressed significantly by EMD (118.19 ± 25.5% Ang II+EMD vs. 136.56 ± 9.4% Ang II, 37 kDa and 102.83 ± 14.38% vs. 169.84 ± 14.24% Ang II, 25 kDa; $P<0.05$) or CA-074Me (90.96 ± 13.52% Ang II+CA-074Me vs. 136.56 ± 9.4% Ang II, 37 kDa and 71.79 ± 7.53% vs. 169.84 ± 14.24% Ang II, 25 kDa; $P<0.05$) (Figure 3.5).

A.



B.

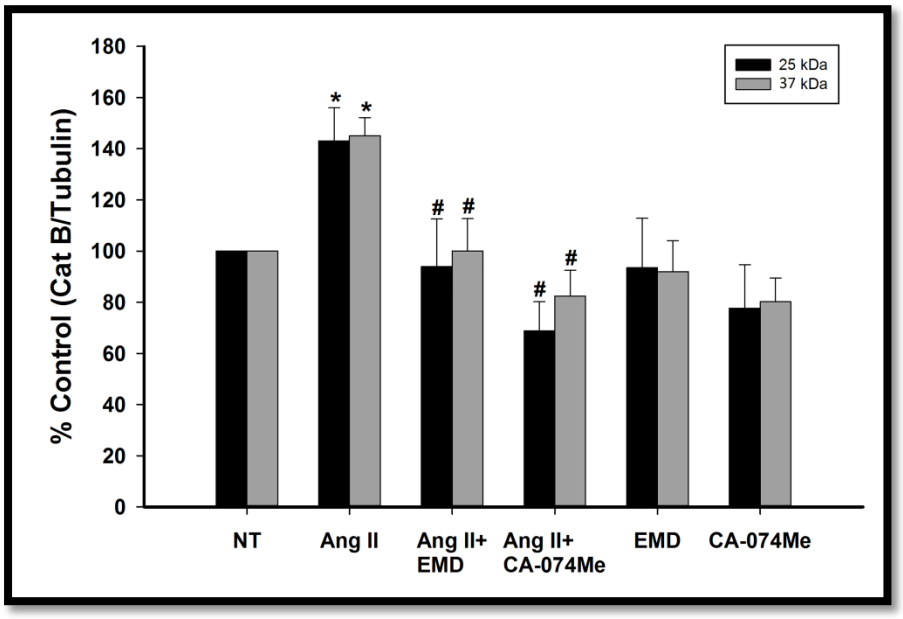
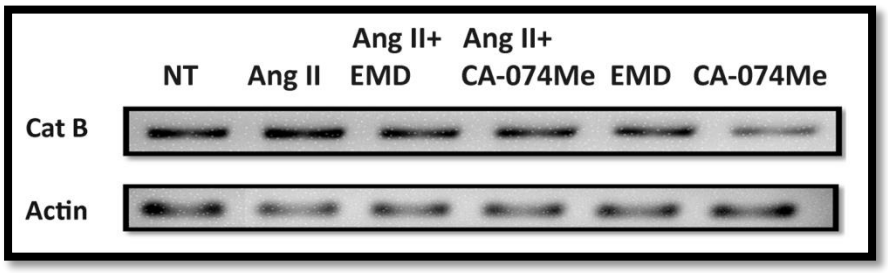


Figure 3.5 Stimulation of the Na^+/H^+ exchanger isoform 1 elicits an increase in Cat B protein expression in H9c2 cardiomyoblasts. A. Representative Western blot of Cat B protein expression in cell lysates of H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=6-7). Immunoblotting was against Cat B (pro form at 37 kDa and active form at 25 kDa) and α-tubulin (50 kDa). **B.** Quantification of relative levels of Cat B protein expression (n=6-7). Results expressed as % of control (α-tubulin) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II

3.1.4.2 Measurement of Cat B mRNA Expression

Our previous findings revealed an increase in Cat B protein expression in the cell lysates upon stimulation of NHE1 with Ang II (Figure 3.5). The increase in protein expression could possibly be as a result of transcriptional changes within the cell. Hence, we stimulated NHE1 using Ang II and looked at its effect on Cat B gene expression by RT-PCR analysis. RT-PCR analysis revealed that Cat B gene expression was significantly elevated at the 10 µM Ang II concentration (288.11 ± 76.72% Ang II vs. 100% control; $P<0.05$) and this increase was regressed significantly by EMD (161.77 ± 37.14% Ang II+EMD vs. 288.11 ± 76.72% Ang II; $P<0.05$) or CA-074Me (197.49 ± 48.82% Ang II+CA-074Me vs. 288.11 ± 76.72% Ang II; $P<0.05$) (Figure 3.6).

A.



B.

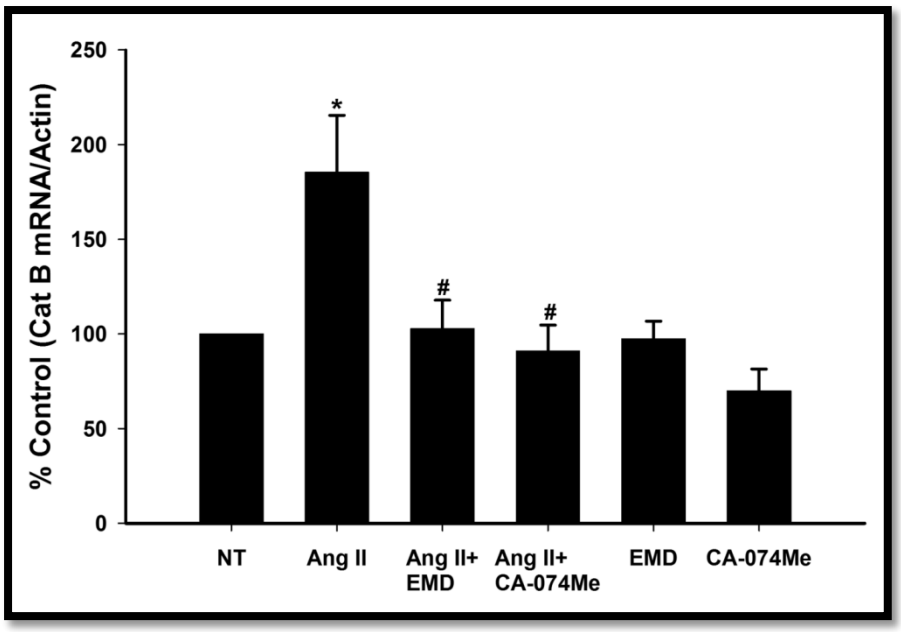


Figure 3.6 Stimulation of the Na^+/H^+ exchanger isoform 1 elicits an increase in Cat B gene expression in H9c2 cardiomyoblasts. A. Representative DNA gel of Cat B mRNA expression in H9c2 cardiomyocytes treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=7). cDNA amplification was against Cat B and β-actin. **B.** Quantification of Cat B mRNA expression in H9c2 cardiomyocytes normalized to β-actin (n=7). Results expressed as % of control (β-actin) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.4.3 Measurement of Cat B Activity Assay

Our previous findings showed that stimulation of NHE1 using Ang II increased the Cat B protein and gene expression significantly (Figures 3.5 and 3.6). Hence, we wanted to further investigate the effect of NHE1 stimulation on the activity of Cat B. In order to determine whether Ang II induces Cat B activity in H9c2 cardiomyocytes, the cells were treated with Ang II, with or without EMD and CA-074Me for 24 hours. Cat B activity assay revealed that Cat B activity was not changed due to Ang II treatment in the cell lysates (Figure 3.7). Analysis of Cat B activity in the conditioned media also revealed no changes in Cat B activity upon activation of NHE1 using Ang II (Figure 3.8). Previous studies have shown that Cat B is optimally active at an acidic pH value (132, 237). It is to be noted that the culture media utilized for our *in vitro* model contained buffers to maintain the pH. The buffers efficiently maintained the pH of the culture media at a neutral value (approximately pH 7.4) which may have prevented the activation of Cat B and hence an inability to see any changes in it. Moreover, it is also possible that the assay was not sensitive enough to detect the activity of Cat B as we did not see any changes in enzyme activity in the positive control as well (data not shown).

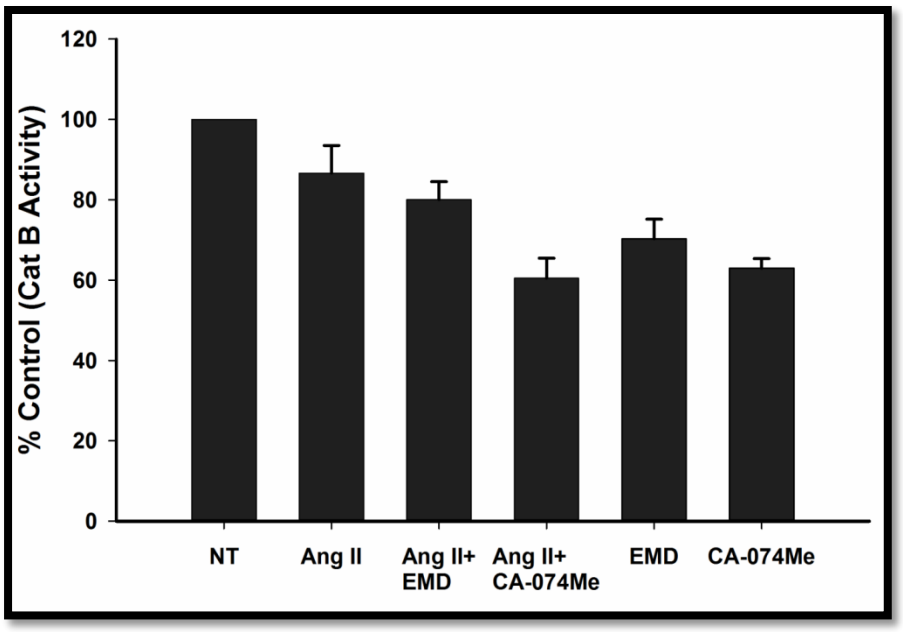


Figure 3.7 Stimulation of the Na^+/H^+ exchanger isoform 1 does not elicits an increase in Cat B enzyme activity in H9c2 cardiomyoblasts. Quantification of Cat B activity in cell lysates of H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=4). Results expressed as % control (non-treated) ± %SEM.

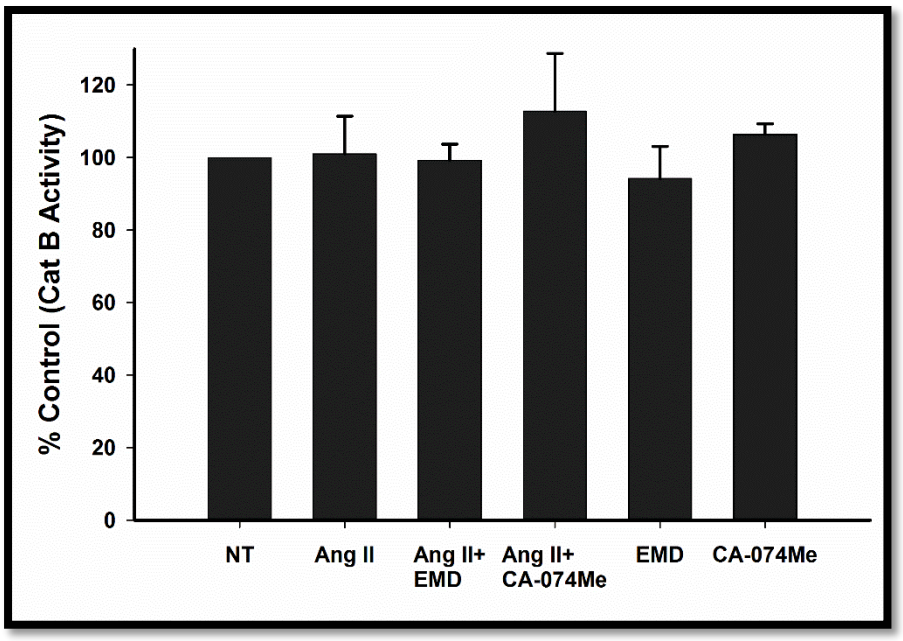


Figure 3.8 Stimulation of the Na^+/H^+ exchanger isoform 1 does not elicits an increase in Cat B enzyme activity in conditioned media from H9c2 cardiomyoblasts. Quantification of Cat B activity in conditioned media from H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=3). Results expressed as % control (non-treated) ± %SEM.

3.1.5 Characterization of Cat B and NHE1 in the NHE1 Induced Hypertrophic Response by Measurement of Cardiac Hypertrophic markers

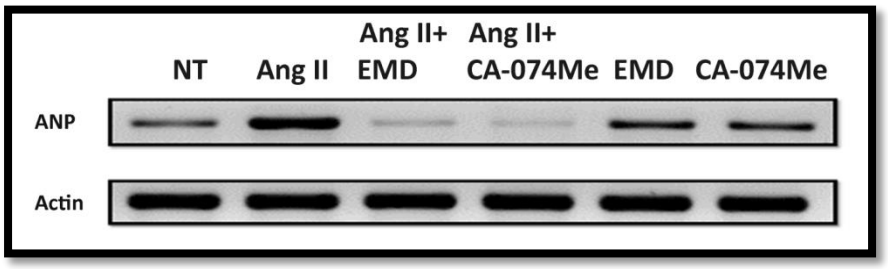
Although Cat B and NHE1 have independently been shown to mediate CH (36, 53, 91, 214), whether Cat B contributes to the hypertrophic response induced by the elevated NHE1 activity remains unclear. In order to investigate the role of NHE1 in the cardiomyocyte hypertrophic cascade we looked at three cardiomyocyte hypertrophic markers namely, ANP mRNA, cell area and protein content. Increase in ANP mRNA and protein synthesis are one of the hallmarks of CH and heart failure (231). Additionally, it was shown that treatment with hypertrophic agonists like ET-1 and Ang II induced an increase in cell surface area (232, 233). Thus, their levels were considered as good markers of cardiomyocyte hypertrophy.

3.1.5.1 Measurement of ANP mRNA

H9c2 cardiomyoblasts stimulated with 10 µM Ang II induced a significant increase in ANP mRNA (393.43 ± 66.29% Ang II vs. 100% control; $P< 0.05$) (Figure 3.9). ANP mRNA expression (59.40 ± 12.24 % Ang II+EMD vs. 393.43 ± 66.29% Ang II; $P< 0.05$) (Figure 3.9) was significantly regressed upon treatment with EMD.

To further confirm the role of Cat B in the NHE1 mediated hypertrophic phenotype, H9c2 cardiomyoblasts stimulated with 10 µM Ang II were pre-treated with CA-074Me. The inhibition of Cat B in cardiomyoblasts expressing active NHE1 was reversed as indicated by the significant reduction in ANP mRNA expression (62.44 ± 13.85% Ang II+CA-074Me vs. 393.43 ± 66.29% Ang II; $P< 0.05$) (Figure 3.9).

A.



B.

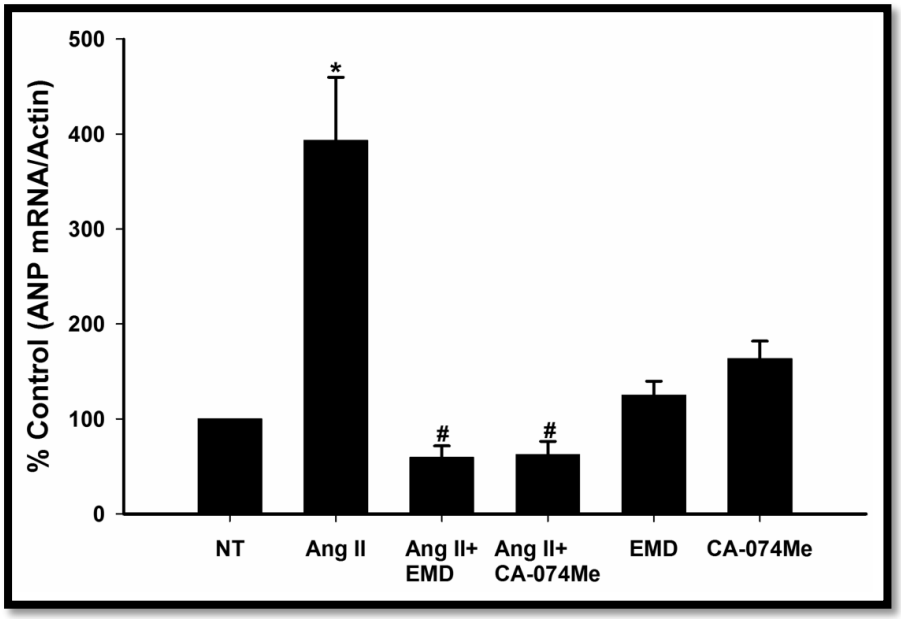


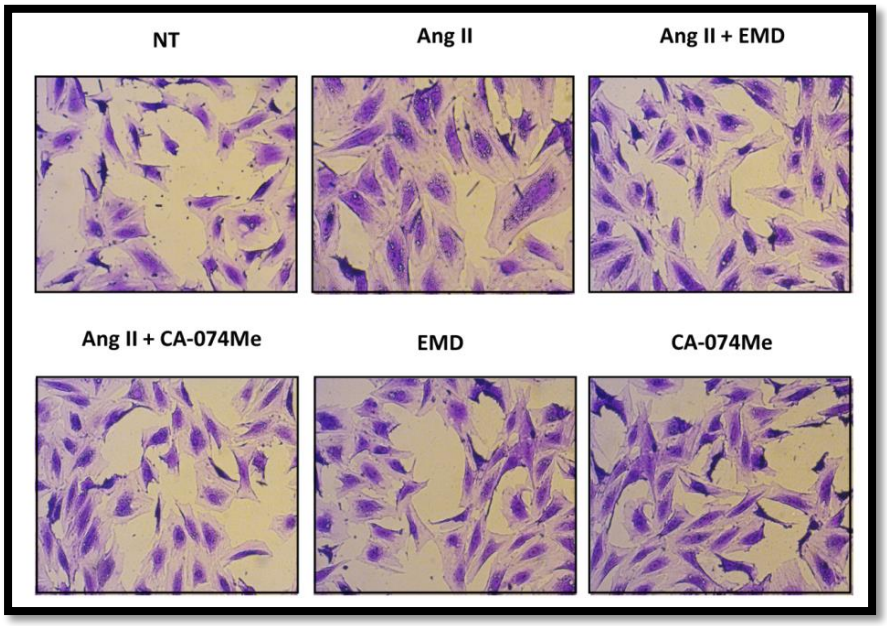
Figure 3.9 Cat B contributes to Na^+/H^+ exchanger isoform 1 -induced cardiomyocyte-hypertrophy in H9c2 cardiomyoblasts. A. Representative DNA gel of ANP mRNA expression in H9c2 cardiomyocytes treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=7). cDNA amplification was against ANP mRNA and β-actin. **B.** Quantification of ANP mRNA expression in H9c2 cardiomyocytes normalized to β-actin (n=7). Results expressed as % of control (β-actin) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.5.2 Measurement of Cell Area

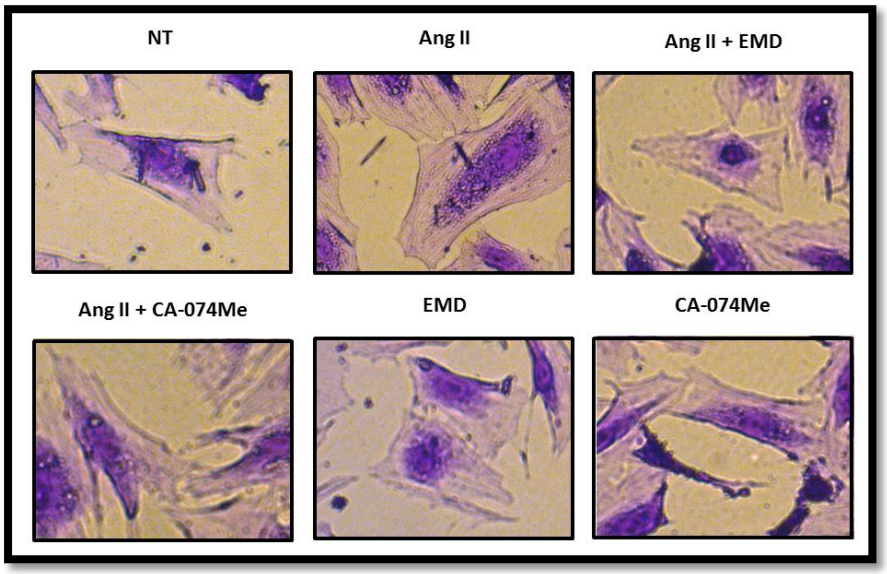
H9c2 cardiomyoblasts stimulated with 10 µM Ang II induced a significant increase in cell surface area (145.9 ± 8.24% vs. 100% control; P< 0.05) (Figure 3.10). Cell surface area (105.3±11.88% Ang II+EMD vs. 145.9 ± 8.24% Ang II; P< 0.05) (Figure 3.10) was significantly regressed upon treatment with EMD.

To further confirm the role of Cat B in the NHE1 mediated hypertrophic phenotype, H9c2 cardiomyoblasts stimulated with 10 µM Ang II were pre-treated with CA-074Me. The inhibition of Cat B in cardiomyoblasts expressing active NHE1 was reversed as indicated by the significant reduction in cell surface area (97.35 ± 9.08% Ang II+CA-074Me vs. 145.9 ± 8.24% Ang II; P< 0.05) (Figure 3.10)

A.


NT
Ang II
Ang II + EMD
Ang II + CA-074Me
EMD
CA-074Me

B.


NT
Ang II
Ang II + EMD
Ang II + CA-074Me
EMD
CA-074Me

C.

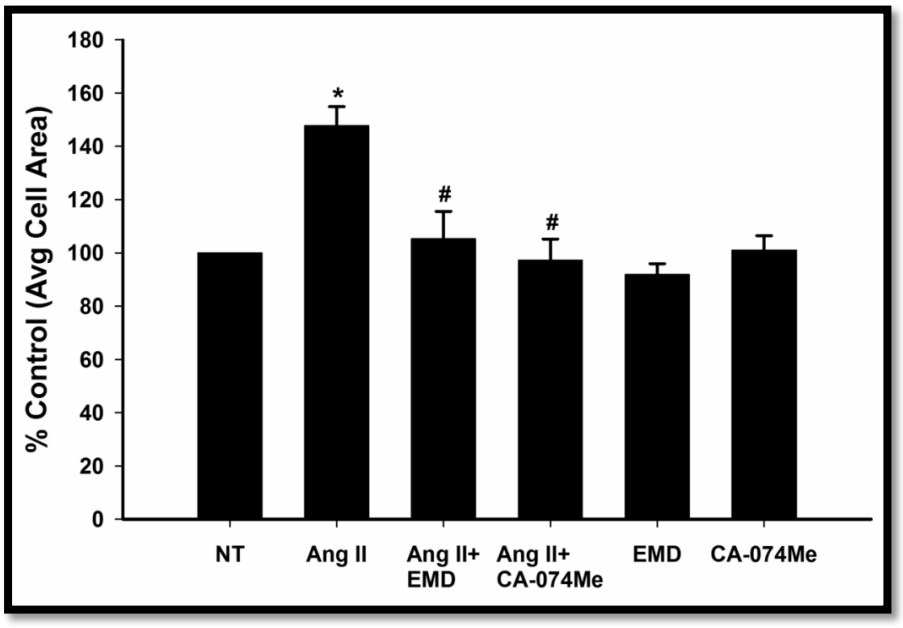


Figure 3.10 Cat B contributes to Na^+/H^+ exchanger isoform 1 -induced cardiomyocyte-hypertrophy in H9c2 cardiomyoblasts. A. Representative crystal violet stained microscopy images of H9c2 cardiomyocytes treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=4) using bright field Carl Zeiss AxioCam at a magnification of 20x. **B.** Images captured at 20x magnification were zoomed to visualize a single H9c2 cardiomyoblast for better comparison between groups. **C.** Cell surface area of at least 30-40 H9c2 cardiomyocytes from 3-4 individual dishes were measured (n=4). Results expressed as % of control (non-treated) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.5.3 Measurement of Protein Content

H9c2 cardiomyoblasts stimulated with 10 µM Ang II induced a significant increase in total protein content (154.04 ± 17.79% Ang II vs. 100% control; $P< 0.05$) (Figure 3.11). Total protein content (96.73 ± 9.58% Ang II+EMD vs. 154.04 ± 17.79% Ang II; $P< 0.05$) (Figure 3.11) was significantly regressed upon treatment with EMD.

To further confirm the role of Cat B in the NHE1 mediated hypertrophic phenotype, H9c2 cardiomyoblasts stimulated with 10 µM Ang II were pre-treated with CA-074Me. The inhibition of Cat B in cardiomyoblasts expressing active NHE1 was reversed as indicated by the significant reduction in total protein content (110.99 ± 13.24% Ang II+CA-074Me vs. 154.04 ± 17.79% Ang II; $P< 0.05$) (Figure 3.11).

Our findings reveal for the first time that NHE1-induced Cat B expression contributes to the hypertrophic response in cardiomyoblasts and that blockage of this effect prevents the NHE1.

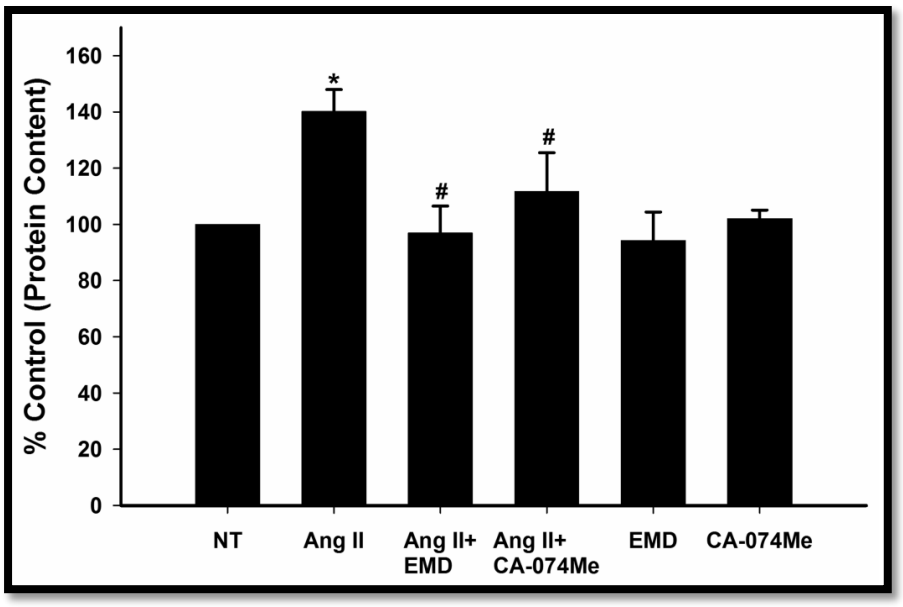


Figure 3.11 Cat B contributes to Na^+/H^+ exchanger isoform 1 induced cardiomyocyte hypertrophy in H9c2 cardiomyoblasts (protein content). Protein content of H9c2 cardiomyocytes expressed as mg/10 x 106 cell. D. Representative crystal violet stained microscopy images of H9c2 cardiomyocytes treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=4). Results expressed as % of control (non-treated) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.6 Translocation of Cat B from the Intracellular to the Extracellular Compartment

3.1.6.1 Intracellular Localization of Lysosomes Using LysoTracker Red Dye

Cat B is localized primarily within the acidic environment of the lysosomes, where they are optimally active and functional to degrade unwanted and damaged proteins (77). The loss of integrity of the lysosomes can cause the release of the Cat B proteases into the cytosol or into the extracellular compartment (86). Previous studies have reported an increase in lysosomal cathepsin protease activity in tissue samples from disease and failing human hearts. Therefore, we aimed to determine the intracellular localization and morphology of lysosomes using the acidotropic probe LysoTracker Red in H9c2 cardiomyoblasts following stimulation with NHE1. H9c2 cardiomyoblasts were stimulated with 10 μM Ang II resulted in a dispersion of the lysosomes as compared to control (Figure 3.12). Inhibition of NHE1 or Cat B showed a more intact structure of the lysosomes (Figure 3.12).

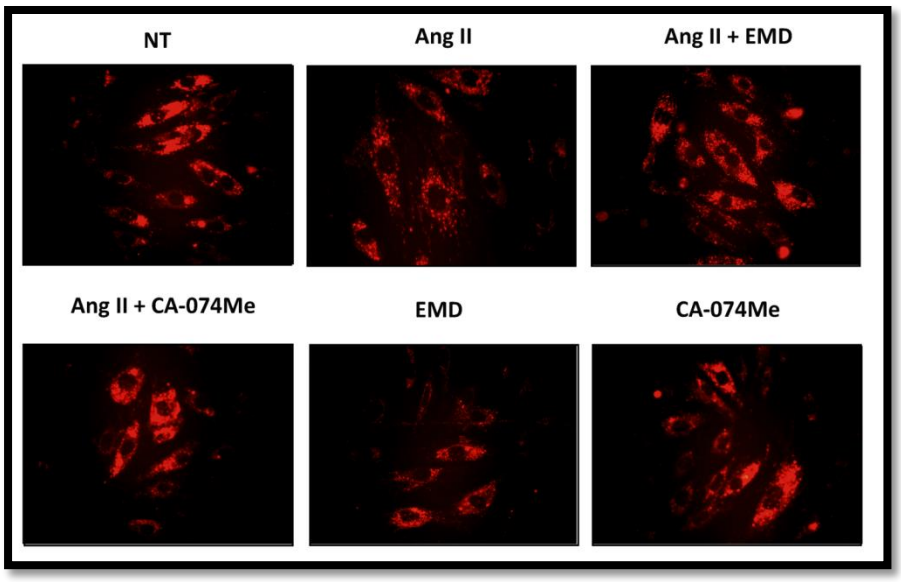
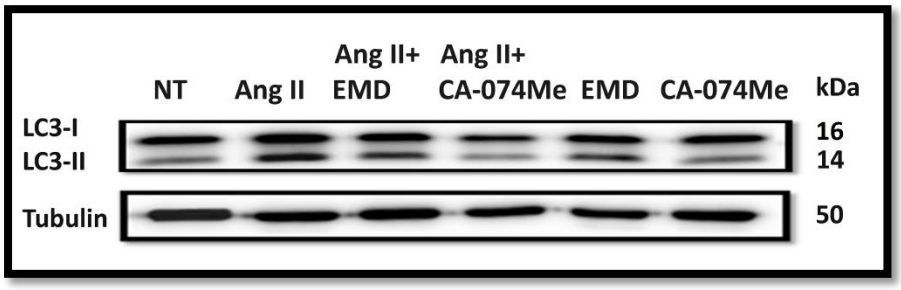


Figure 3.12 Stimulation of the Na^+/H^+ exchanger isoform 1 causes the loss of lysosomal integrity in H9c2 cardiomyoblasts which is protected by Cat B inhibition. Representative images of H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours using fluorescent microscope OlympusIX70 at 20x magnification (n=3). The cells were stained with Lysotracker Red dye.

3.1.6.2 Measurement of LC3-II Protein Expression in Cell Lysates

Our previous findings revealed an increase in dispersion of lysosomes upon stimulation of NHE1 and this effect seemed to be protected by the inhibition of Cat B or NHE1 (Figure 3.12). Cathespins have previously been demonstrated to be important in maintaining the autophagy- lysosomal pathway activated in response to stress (131). Previous studies have revealed that autophagy plays an important role in the pathogenesis of CVDs (239). Moreover, NHE1 has also been implicated to promote autophagy (240). Microtubule‐associated protein light chain 3-II (LC3-II) is widely used to monitor autophagy. The amount of LC3‐II is correlated with the number of autophagosomes (130, 241), which have been shown to cause the release of the Cat B proteases into the cytosol or into the extracellular compartment (59). The levels of LC3-II protein was measured by immunoblot analysis in H9c2 cardiomyoblasts treated with 10 µM Ang II, with or without the NHE1 or Cat B inhibitors. Our results revealed that LC3-II protein expression, appearing at 14 kDa, was significantly elevated following stimulation with 10 µM Ang II (204.38 ± 49.83% Ang II vs. 100% control; $P<0.05$) (Figure 3.13). This effect that was significantly regressed upon treatment with EMD (101.35 ± 16.32% Ang II+EMD vs. 204.38 ± 49.83% Ang II.; $P<0.05$) (Figure 3.13) or CA-074Me (91.79 ± 22.59% Ang II+CA-074Me vs. 204.38 ± 49.83%Ang II.; $P<0.05$) (Figure 3.13). Our results demonstrate that stimulation of NHE1 in H9c2 cardiomyoblasts with Ang II induces a significant increase in the autophagic marker LC3-II and this effect is regressed by inhibition of NHE1 or Cat B.

A.



B.

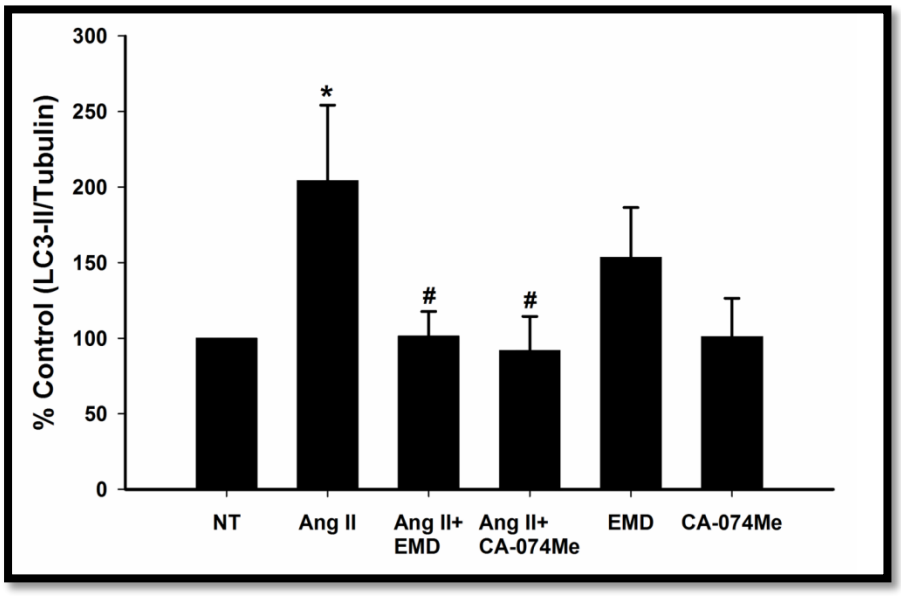


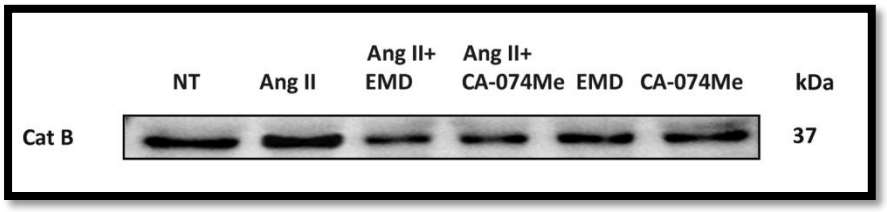
Figure 3.13 Na^+/H^+ exchanger isoform 1 -induced Cat B expression elicits an increase in autophagy in H9c2 cardiomyoblasts. A. Representative Western blot of LC3 protein expression of H9c2 cardiomyoblasts treated with Ang II in the presence and absence of EMD or CA-074Me for 24 hours (n=5). Immunoblotting was against LC3-I/II (14 and 16 kDa). **B.** Quantification of relative levels of LC3-II protein expression (n=5). Results are expressed as a % of control (α-tubulin) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.6.3 Measurement of Cat B in the Media

Our previous findings indicate that stimulation of NHE1 using Ang II causes a loss of integrity of the lysosomes (Figure 3.12) and increases the number of autophagosomes (Figure 3.13). Moreover, activation of NHE1 has also shown to increase the secretion of Cat B into the extracellular spaces by mediating lysosomal redistribution to the cell peripheries (132, 136). Hence, we aimed to determine the protein levels of Cat B in the conditioned media obtained from H9c2 cardiomyoblasts treated with Ang II, with or without the NHE1 or Cat B inhibitors. Immunoblot analysis revealed that Cat B protein levels were significantly elevated following stimulation with Ang II (136.54 ± 9.85% Ang II vs. 100% control; $P<0.05$) (Figure 3.14), an effect that was significantly regressed upon treatment with EMD (81.11 ± 13.06% Ang II+EMD vs. 136.54 ± 9.85% Ang II; $P<0.05$) (Figure 3.14). Similarly, Cat B protein expression in the media was significantly regressed when pre-treated with CA-074Me (94.32 ± 12.96% Ang II+CA-074Me vs. 136.54 ± 9.85% Ang II; $P<0.05$) (Figure 3.14). It is to be noted that the immunoblot analysis revealed only the preform of Cat B in the media unlike that seen in the cell lysates. The presence of only the pro form of Cat B in the culture media can be explained by the presence of buffers in the media which maintain a neutral pH. Absence of optimal pH conditions can prevent the activation of pro Cat B and hence explain our inability to see the active form of the enzyme in the media (237). Moreover, it is also possible that the active form of Cat B is being degraded in the media as the half-life of Cat B is around 14 hours (60), which is less than our treatment condition of 24 hours. Our results demonstrate that stimulation of NHE1

with Ang II in H9c2 cardiomyoblasts induces an increase in Cat B protein expression in the extracellular compartment, which is inhibited by NHE1 or Cat B inhibition.

A.



B.

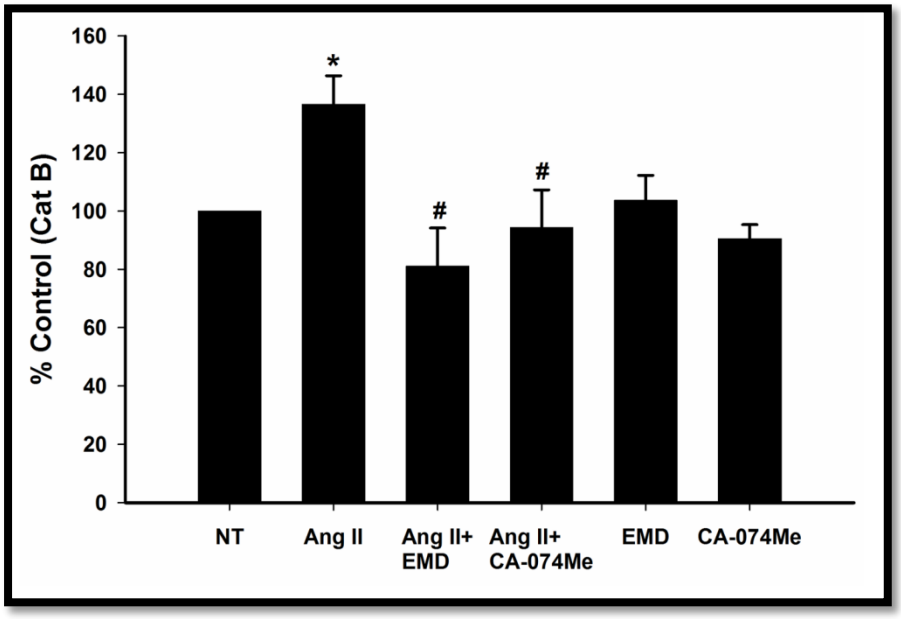


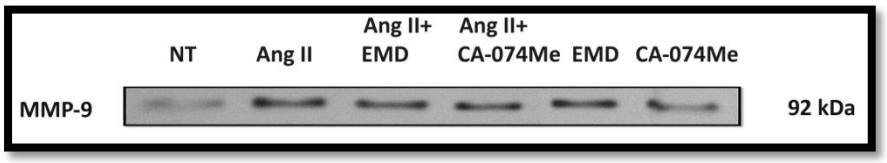
Figure 3.14 Stimulation of the Na^+/H^+ exchanger isoform 1 elicits an increase in Cat B protein in the extracellular environment of H9c2 cardiomyoblasts. A. Representative Western blot of Cat B protein expression in the conditioned media of H9c2 cardiomyoblasts treated with Ang II in the presence or absence of EMD or CA-074Me for 24 hours (n=5). Immunoblotting was against Cat B (pro form at 37 kDa). **B.** Quantification of the relative levels of Cat B protein expression in the conditioned media (n=5). Results are expressed as a % of control (non-treated) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.6.4 Measurement of MMP-9 Gelatinolytic Activity and Protein Expression in the Conditioned Media

3.1.6.4.1 Measurement of MMP-9 Gelatinolytic Activity in the Conditioned Media

Previous reports have demonstrated that Cat B has the ability to activate pro-MMP-9, which can result in further ECM degradation (218). MMP-9 activity was also increased in CCL39 cells upon the stimulation of NHE1 with phenylephrine (222). Interestingly, Cat B and MMP-9 have been demonstrated to interact with NHE1 to promote ECM degradation in breast cancer (133). Hence, we aimed to determine the MMP-9 gelatinolytic activity in the conditioned media obtained from H9c2 cardiomyoblasts treated with Ang II, with or without the NHE1 or Cat B inhibitors. Gelatin zymography revealed that MMP-9 gelatinolytic activity was significantly elevated following stimulation with 10 µM Ang II (134.37 ± 5.61% Ang II vs. 100% control; $P<0.05$) (Figure 3.15), an effect that was significantly regressed upon stimulation with EMD (93.74 ± 10.79% Ang II+EMD vs. 134.37 ± 5.61 % Ang II vs.; $P<0.05$) (Figure 3.15). Similarly, MMP-9 activity was significantly regressed when pre-treated with CA-074Me (85.24 ± 13.17% Ang II+CA-074Me vs. 134.37 ± 5.61 % Ang II vs.; $P<0.05$) (Figure 3.15). Our results indicate that stimulation of NHE1 with Ang II in H9c2 cardiomyoblasts induces an increase in MMP-9 gelatinolytic activity which is inhibited by NHE1 or Cat B inhibition.

A.



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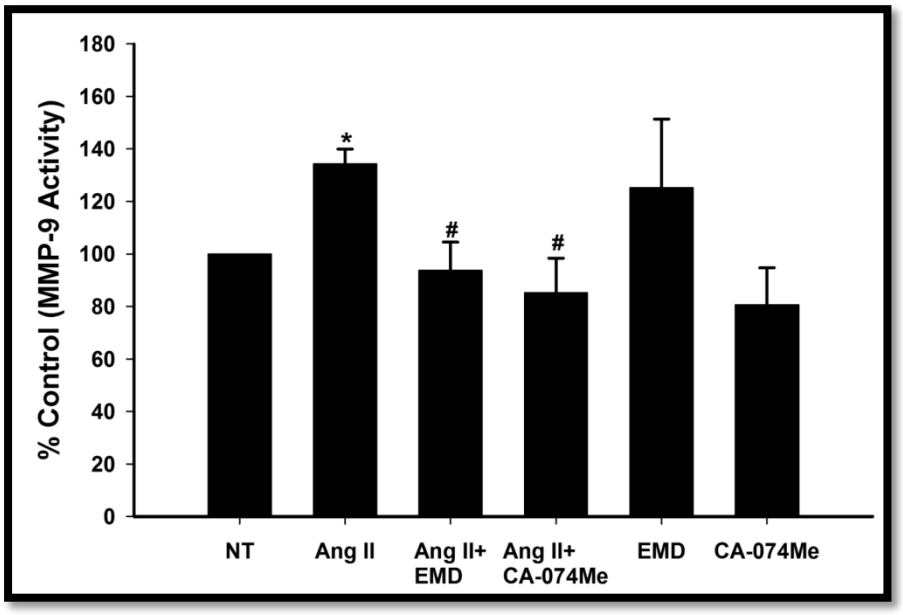
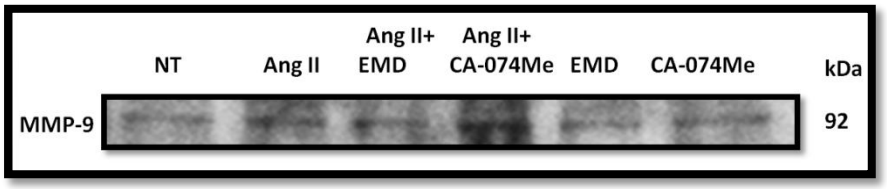


Figure 3.15 Na^+/H^+ exchanger isoform 1 induced Cat B expression contributes to MMP-9 gelatinolytic activity in the extracellular environment of H9c2 cardiomyoblasts. A. Representative zymogram of MMP-9 gelatinolytic activity in conditioned media treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=4). Bands were visualized at 92 kDa corresponding to MMP-9. **B.** Quantification of relative levels of MMP-9 bands (n=4). Results are expressed as % of control (non-treated) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.6.4.2 Measurement of MMP-9 Protein Expression in the Conditioned Media

It had previously been reported that the levels of MMP-9 are increased in models of heart failure. Hence we looked at the MMP-9 protein quantity in the conditioned media. Our Immunoblot analysis revealed that the stimulation of NHE1 did not induce any changes in the MMP-9 protein levels conditioned media (Figure 3.16)**.**

A.



B.

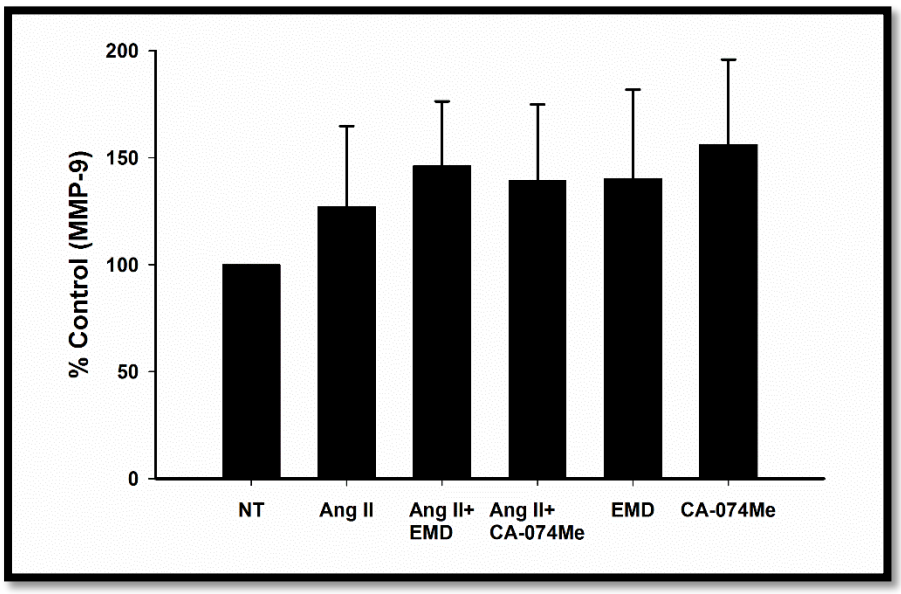


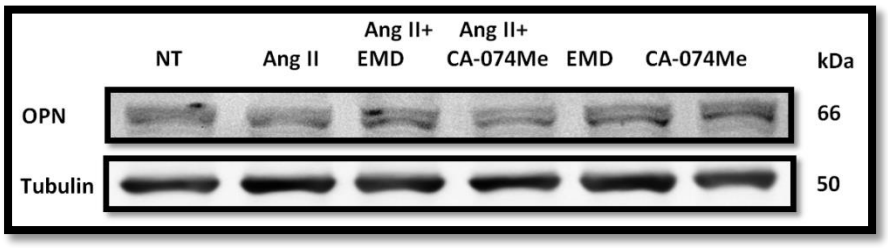
Figure 3.16 Na^+/H^+ exchanger isoform 1 induced Cat B expression does not induce MMP-9 protein expression in the extracellular environment of H9c2 cardiomyoblasts. **A.** Representative Western blot of MMP-9 protein expression in conditioned media of H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=6). Immunoblotting was against MMP-9 (92 kDa). **B.** Quantification of relative levels of MMP-9 protein expression in the conditioned media (n=6). Results expressed as % of control (non-treated) ± %SEM.

3.1.7 Investigation of Signaling Pathways That Contribute Towards Cardiomyocyte Hypertrophy

3.1.7.1 Measurement of OPN Protein Expression

Previous reports have shown that OPN modulates Ang II induced cardiac fibrosis in mice hearts (242) Moreover, OPN has also been shown to be cause cardiomyocyte hypertrophy in cooperation with NHE1 (51, 52). Previous report have demonstrated that certain MMPs have a cleavage site on OPN (243). We have seen previously that Ang II causes a significant increase in MMP-9 activity (Figure 3.15). Hence, we wanted to determine whether this increase in MMP-9 activity had any effect on OPN protein expression. Our findings revealed that the OPN protein expression remained unchanged upon treatment with Ang II (Figure 3.17).

A.



B.

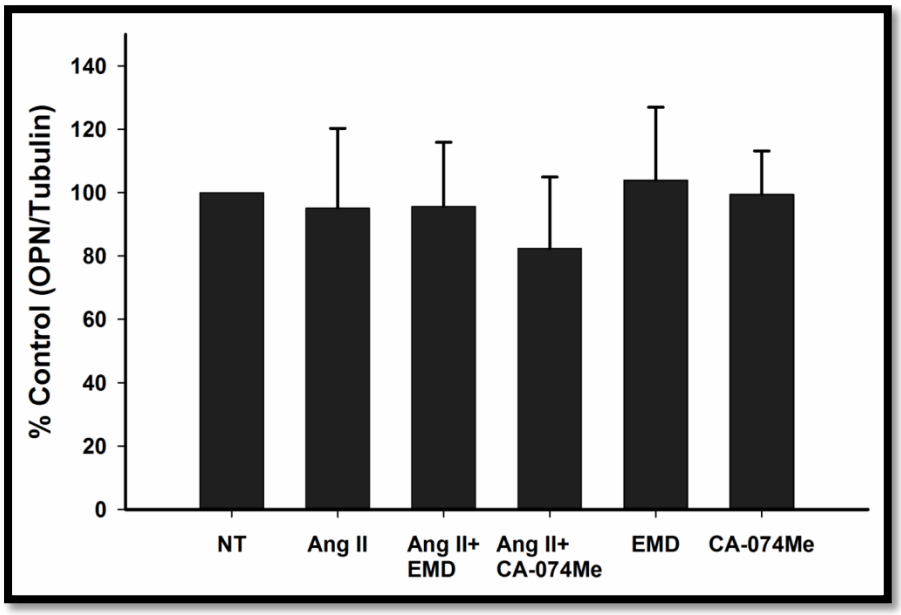


Figure 3.17 Na^+/H^+ exchanger isoform 1 induced Cat B expression does not induce OPN protein expression in H9c2 cardiomyoblasts. A. Representative Western blot of OPN protein expression in cell lysates of H9c2 cells treated with Ang II, with or without EMD and CA-074Me for 24 hours (n=3). Immunoblotting was against OPN (66 kDa) and α-tubulin (50 kDa). **B.** Quantification of relative levels of OPN protein expression (n=3). Results expressed as % of control (α-tubulin) ± %SEM. *$p < 0.05$ vs. control, # $p < 0.05$ vs. Ang II.

3.1.8 Investigation of Apoptosis in the Cat B Induced Cardiomyocyte Hypertrophic Response; Measurement of Apoptotic Markers; Bcl2 and Bax mRNA

Cat B and NHE1 have independently been shown to be involved in apoptosis (123, 124) (244, 245). Moreover, previous report have demonstrated an increase in the apoptotic marker Bax mRNA and a decrease in the anti-apoptotic marker Bcl-2 mRNA in H9c2 cardiomyoblasts treated with Ang II (216). Hence, we wanted to see the effect of our treatment on the apoptotic markers. Our results revealed no change in the apoptotic markers which may be due to the different concentrations used in both studies (Figure 3.18, 3.19).

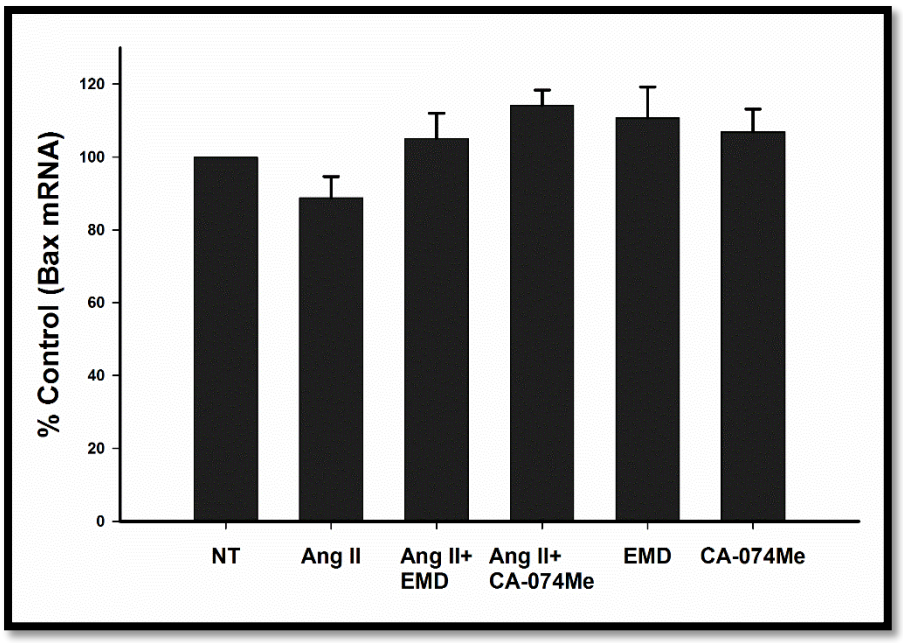


Figure 3.18 Na^+/H^+ exchanger isoform 1 induced Cat B expression does not induce Bax mRNA in H9c2 cardiomyoblasts. Quantification of BAX mRNA expression in H9c2 cardiomyocytes normalized to β-actin (n=5). Results expressed as % of control (β-actin) ± %SEM.

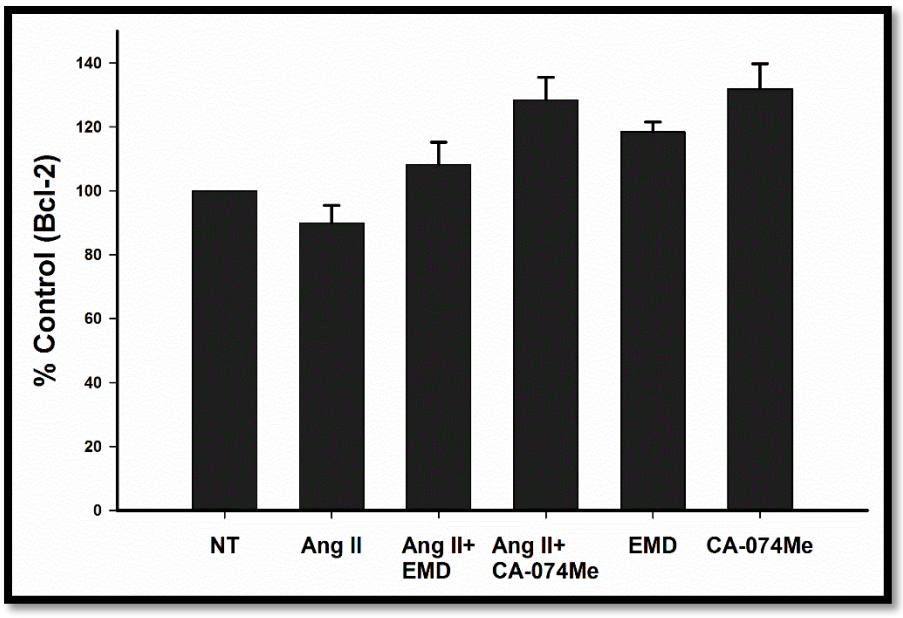


Figure 3.19 Na^+/H^+ exchanger isoform 1 induced Cat B expression does not induce Bcl-2 mRNA in H9c2 cardiomyoblasts. Quantification of Bcl-2 mRNA expression in H9c2 cardiomyocytes normalized to β-actin (n=5). Results expressed as % of control (β-actin) ± %SEM. *p < 0.05 vs. control, # p < 0.05 vs. Ang II.

3.2 *In vivo* Studies to Investigate Cat B Protein Expression in Hearts from Transgenic Mice Overexpressing NHE1 (K-Line)

3.2.1 Immunohistochemistry: Cat B Protein Expression in Transgenic Mice Overexpressing NHE1 (K-line)

To confirm our *in vitro* studies, Cat B protein expression was examined in heart sections from wild type mice and mice overexpressing NHE1 (53). The results revealed increased Cat B staining in hearts (arrows indicate increased Cat B staining) from mice overexpressing NHE1 as compared to control hearts (Figure 3.20). Our results indicate that increased expression of NHE1 induces Cat B protein expression *in vivo*.

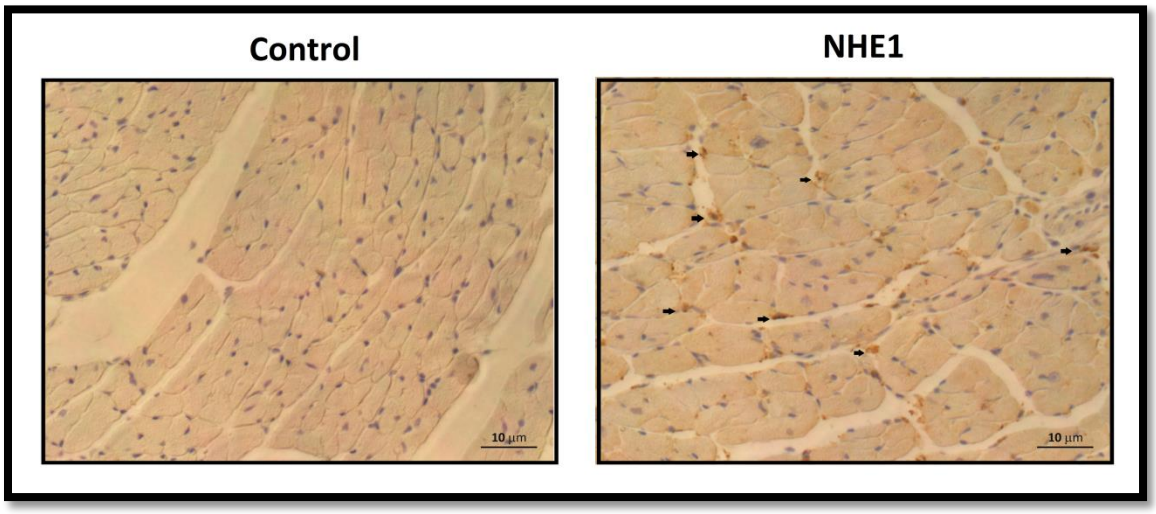


Figure 3.20 *In vivo* immunohistochemical staining for Cat B in transgenic mice overexpressing NHE1 (K-line). Protein expression of Cat B in the myocardium of wild type mice and mice overexpressing NHE1 (K-line). Images were acquired through NIKON bright field microscope at a magnification of 40x. Scale bar=10 µm.

Chapter 4: DISCUSSION AND CONCLUSION

Progression of the heart to failure is primarily caused due to significant remodeling of the myocardium. Remodeling occurs as a result of an imbalance between the synthesis and degradation of the ECM. This imbalance can occur as a result of increased proteolytic activities of proteases such as cathepsins and MMPs and decrease in the levels of TIMPs (77, 214). Cat B is active under conditions of low pH (86, 218, 237). A previous report has demonstrated that pericellular acidification redistributed the Cat B containing lysosomes to the cell surface and caused the secretion of Cat B into the extracellular compartment (86). An overactive NHE1, such as that which is seen in conditions of CH (50, 215, 246, 247), contributes to pericellular acidification. Overactive NHE1 caused a drop in extracellular pH values, which was shown to activate Cat B in a breast cancer *in vitro* model (132). Once into the extracellular compartment, Cat B can degrade the ECM (68) and facilitate further ECM degradation by activating other proteases such as MMP-9 (217, 218). MMP-9 activity has been shown to be increased in various models of heart failure (43, 49, 165, 219). Interestingly, Cat B and MMP-9 were shown to directly interact with NHE1 and cause ECM degradation (133). Whether Cat B participates in cardiac remodeling by mediating ECM degradation in cooperation with MMP-9 due to extracellular pH changes caused by the NHE1 remains unclear. Moreover, Cat B and NHE1 have independently been shown to be involved in apoptosis (123, 124, 244, 245). Whether Cat B and NHE1 work in cooperation to induce apoptosis remains unknown.

Our project aimed to delineate the role of Cat B in the NHE1 induced cardiomyocyte hypertrophic response. To investigate the effects of Cat B in the NHE1 induced cardiomyocyte hypertrophic response we used both an *in vitro* model as well as an *in vivo* model. Ang II was used to stimulate NHE1 activity in H9c2 cardiomyoblasts. Changes in the Cat B gene and protein expression as well as activity were analyzed in the presence and absence of Cat B or NHE1 inhibitor. We also looked at the protein levels of Cat B in transgenic mice harboring the active form of NHE1. Furthermore, we also looked at the effects of NHE1 induced cardiomyocyte hypertrophy on activation of autophagy and the extracellular Cat B protein expression and MMP-9 activity. We also assessed the cardiomyocyte hypertrophic response by measuring the well-established cardiomyocyte hypertrophic markers ANP mRNA, cell area and protein content. Our findings have several implications:

4.1 Ang II induces an increase in NHE1 activity without an increase in its protein expression

Previous reports have demonstrated that NHE1 activity increases upon stimulation with Ang II in ventricular myocytes (227). Previous reports have suggested that the Ang II stimulates the activity of NHE1 by the activation of $G\alpha_q$ protein coupled receptors (section 1.5.3.2). Activation of $G\alpha_q$ signaling then activates downstream signaling cascade (Figure 1.4). Ang II, acting via the AT1 receptor ($G\alpha_q$), induces an increase in intracellular calcium that then interacts with calmodulin (CaM). The Ca^{2+}/CaM complex directly or indirectly

activates NHE1 and phosphorylates calmodulin kinase II (CaMKII), which then regulates the NHE1 activity (248). In our study, the NHE1 activity assay revealed that the NHE1 activity was significantly increased upon stimulation with 100 nM Ang II for 18 hours and this effect was significantly regressed by using EMD, confirming previous reports (51, 52) (Figure 3.1). Based on our findings and previous reports, we used Ang II to induce NHE1 activity in our study in H9c2 cardiomyoblasts.

In order to assess if stimulation with Ang II also induces NHE1 protein expression, we treated the H9c2 cells with 10 µM Ang II for 24 hours in the presence and absence of Cat B or NHE1 inhibition. Immunoblot analysis revealed that Ang II or the inhibition of Cat B did not have any changes in the NHE1 protein levels upon stimulation with Ang II in H9c2 cardiomyoblasts (Figure 3.2). This is in accordance with what has been previously reported. A study demonstrated that MDCK cell treated with 1 nM Ang II did not stimulate an increase in the protein expression of NHE1 whereas, the treatment was sufficient to induce an increase in NHE1 activity (248).

4.2 Stimulation of NHE1 Induces the Expression of Cat B

Cat B and NHE1 have independently been shown to mediate CH (36, 53, 91, 214). Moreover, the activation NHE1 has been shown to activate Cat B in a breast cancer model (132). Hence, we investigated the effect of overactive NHE1 (stimulated using Ang II) on the Cat B protein expression in H9c2 cardiomyoblasts. Immunoblot analysis revealed that

Cat B protein expression in its pro and active forms were significantly increased in H9c2 cardiomyoblasts upon stimulation with 10 µM Ang II for 24 hours (Figure 3.3).

The pro region of the pro Cat B enzyme is cleaved off to liberate the mature or active form of Cat B which is of 30 kDa. The pro Cat B can be converted into active Cat B by exposure to low pH, or to Cat D (aspartic protease) (237). Pro Cat B can also convert to its active form by self-cleavage (74-76). Furthermore, the 30 kDa active form can be further processed by cleavage of its dipeptide bonds and liberation of its heavy chain (25 kDa) and a light chain (5 kDa). The heavy and light chains forms are linked together cysteine–cysteine bonds (60). It is to be noted that Cat B pro form is indicated to be enzymatically inactive and requires an acidic pH to become active. Although it shows maximum activity at an acidic pH, it has been found that Cat B retains significant proteolytic activity at neutral pH as well (88).

Apart from investigating the **concentration** of Ang II at which it induces maximum Cat B expression we also wanted to know the **time point** for the same. For this we used a concentration of 10 µM Ang II (Figure 3.3) at different time points (section 2.2 (c)). Immunoblot analysis revealed that Cat B protein expression was maximally increased at 24 hours (Figure 3.4). This is in accordance with a recent study, where they induced Cat B protein expression in H9c2 cardiomyoblasts using Ang II at a 24 hour time point (216). Hence, for our study we used a concentration of 10 µM Ang II for 24 hours to induce Cat B protein expression. The H9c2 cell line shows a similar hypertrophic response as the primary cardiomyoblasts and hence is used as a model for *in vitro* study of CH (225).

Furthermore, our study demonstrated that the Cat B mRNA expression was significantly increased in H9c2 cardiomyoblasts upon stimulation with 10 µM Ang II for 24 hours (Figure 3.6). This increase in Cat B protein and gene expression was regressed upon inhibition of NHE1 or Cat B (Figure 3.5 and 3.6). A study done on bone marrow derived macrophages demonstrated that CA-074Me has the ability to affect transcription factor NFATc1 through down regulation of c-FOS. Auto-amplification of NFATc1 is necessary for the transcription of genes such as those of cathepsins. This study demonstrated that CA-074Me was able to inhibit the NFATc1 mRNA via inhibiting c-FOS resulting in the inhibition of transcription. This study might explain the inhibition of Cat B mRNA upon using CA-074Me in our model (249).

To confirm our *in vitro* studies, we investigated Cat B protein expression in an *in vivo* model. Immunohistochemical analysis done on heart sections from wild type mice and mice overexpressing NHE1 (53) revealed increased Cat B staining in hearts from mice overexpressing NHE1 as compared to control hearts (Figure 3.20). Our results indicate that the activity and expression of NHE1 induces Cat B protein levels *in vitro* and *in vivo* respectively (Figure 3.5 and 3.20). These findings are in accordance with Wu *et al*'s recent findings that demonstrated an increase in Cat B upon stimulation with Ang II (216). Moreover, Wu *et al* demonstrated that Cat B deficiency attenuated cardiac remodeling in response to pressure overload (216). Whether the effects seen in Wu *et al*'s study were as a result of activation of NHE1 have not been investigated. Previous reports have showed that pressure overload can lead to the activation of NHE1 (228). The results obtained in our study from NHE1 transgenic mice and the inhibition of NHE1 in H9c2 cardiomyoblasts

show for the first time that Ang II stimulation of Cat B is mediated by NHE1. Hence, indirect inhibition of NHE1 by inhibiting Cat B may prove to be a useful strategy to prevent CH.

Apart from Cat B, various other forms of cathepsins have been suggested to be essential for developing a hypertrophic phenotype. A previous study showed that in cultured neonatal rat cardiomyocytes the gene and protein levels of Cat S, B, and L were upregulated (36). Similarly, Cat S has been shown to be increased in the failing rat myocardium in association with hypertension (36). Levels of Cat S were also increased in hearts extracted from patients with heart failure (36). Loss of Cat L promoted CH upon phenylephrine-induced CH *in vitro* (131). Another study reported that MI induced by left coronary artery ligation in wild-type rats caused rapid Cat L activation in the myocardium and its deficiency contributed to diminished function and adverse remodeling late post-MI. Moreover, Cat S seems to play a role in cardiac maintenance as well since its deletion exacerbated Ang II-induced cardiac inflammation (101). Although many forms of cathepsin have been suggested to contribute to the hypertrophic phenotype, in our report we focused on Cat B, which is activated by an acidic pH (86, 132). Whether the various forms of cathepsins are simultaneous up regulated in a model of hypertrophy or the interplay between the various forms of cathepsins remains unknown. Further experiments are needed in order to find out the role of other forms of cathepsins that are implicated in CH such as Cat S, K and L.

4.3 Stimulation of NHE1 Induces Cat B Mediated Cardiomyocyte Hypertrophy

Despite numerous reports that indicate the involvement of Cat B in the hypertrophic response, the exact mechanism by which it induces cardiomyopathy remains unknown. NHE1 has also been shown to mediate CH (36, 53, 91, 214, 250, 251). Whether active NHE1 contributes to the Cat B mediated hypertrophic response I still not known. *In vitro*, pharmacological Cat B inhibition using CA-074Me suppressed the cardiomyocytes hypertrophy by inhibiting the ASK1/JNK pathway (216). *In vivo* Cat B deficiency attenuated the cardiac remodeling process in response to pressure overload (216). Similarly, Cat B inhibition using CA-074Me significantly attenuated cardiac dysfunction, reduced cardiomyocyte size and cardiac fibrosis *in vivo* (96). In patients with dilated cardiomyopathy, Cat B has been demonstrated to be elevated (91).

Investigation of the cardiomyocyte hypertrophic cascade revealed a significant increase in all three cardiomyocyte hypertrophic markers (Figure 3.9, 3.10, 3.11) confirming previous reports, which have demonstrated that overactive NHE1 induces cardiomyocyte hypertrophy (54, 55, 252). This increase was significantly regressed upon inhibition of NHE1 or Cat B (Figure 3.9, 3.10, 3.11). These findings are in accordance with Wu *et al*'s recent findings that demonstrated a decrease in the cardiomyocyte hypertrophy upon inhibition of Cat B using CA-074Me (216). Our findings reveal for the first time that Cat B expression contributes to the hypertrophic response in cardiomyoblasts and that blockage of this effect prevents the NHE1 induced hypertrophic response. The results from our study provide evidence that NHE1 induced cardiomyocyte hypertrophic response is mediated by Cat B. Hence, inhibition of Cat B may prove to be useful in regressing CH.

4.4 Stimulation of NHE1 Causes the Dispersion of Lysosomes Which is Protected by the Inhibition of Cat B

Hydrogen ions (H^+) or protons are important for the regulation of many physiological process like DNA synthesis, cell cycle, cell volume, cell growth, and cell proliferation. The levels of proton are controlled by exchangers present on the cell membranes such as the NHEs, Na^+-dependent and -independent HCO^{3-}/Cl^- exchangers and the H^+/lactate co transporter (174). It has been demonstrated that lysosomes were redistributed to the cell periphery upon a drop in the extracellular pH values caused by an overactive NHE1 (132, 133). (86, 134, 135). This can subsequently cause the secretion of lysosomal contents into the ECM as it has been shown for Cat B which is primarily present within the lysosomes (86). Moreover, acidic extracellular pH caused the redistribution of the lysosome to the cell periphery which paralleled cathepsin B secretion. Interestingly this effect was blocked with several broad and specific NHE inhibitors (136). Similarly, in our study we demonstrated that H9c2 cardiomyoblasts stimulated with Ang II showed more dispersed lysosomal vesicles as compared to control (Figure 3.12). This effects seemed to be protected upon inhibition of the NHE1 or Cat B, where the H9c2 cardiomyoblasts showed a more intact structure of the lysosomes (Figure 3.12). The loss of integrity of the lysosomes can cause the release of the Cat B proteases into the cytosol or into the extracellular compartment (86).

4.5 Stimulation of NHE1 Causes the Activation of Autophagy Which is regressed by the Inhibition of Cat B

The autophagy–lysosomal system and the ubiquitin–proteasome system (UPS) are responsible for majority of the protein degradation in eukaryotic cells (128). In the cardiovascular system there is a fine balance between the two systems to maintain homeostasis of proteins and organelles (129). The UPS targets small and short lived protein by tagging them with ubiquitin (129). In contrast, the autophagy–lysosomal pathway targets long-lived bulky proteins that are defected or damaged under stress (130). Impaired or dysregulated protein degradation in the heart has recently been recognized as a potential major contributor to cardiac disease. Cathepsins an important lysosomal protein–processing enzyme that play an important role in maintaining the autophagy- lysosomal pathway activated in response to stress (131). The final step of the autophagy–lysosomal pathway is the fusion of an autophagosome with a functioning lysosome, and an imbalance of protein homeostasis by dysfunction of this system may lead to pathological hypertrophy and dysfunction. It has been reported that lysosomal proteases such as cathepsins play a major role in cardiac protein turnover, but the mechanism is not well understood. Moreover, NHE1 mediated changes in pH has also been shown to promote autophagy in neurons (240). Hence, we aimed to determine the levels of LC3-II protein. Our results revealed that LC3-II protein expression was significantly elevated following stimulation with Ang II (Figure 3.13) and this effect that was significantly regressed upon treatment with EMD or CA-074Me (Figure 3.13). Our results demonstrate that stimulation of NHE1

induces a significant increase in autophagy and this is regressed by inhibition of NHE1 and Cat B.

4.6 Stimulation of NHE1 Induces an Increase in Cat B Protein in the Extracellular Compartment

Decreased extracellular pH values can cause increased redistribution of lysosomes to the cell surface (86) which may be accompanied by the fusion of an autophagosome with a functioning lysosome (131). This can subsequently cause the secretion of Cat B, into the ECM (86). Our previous findings indicate that stimulation of NHE1 using Ang II causes increased dispersion of lysosomes (Figure 3.12) and increases the number of autophagosomes (Figure 3.13). Hence, we wanted to investigate the Cat B protein aimed to determine the protein levels of Cat B in the conditioned media obtained from H9c2 cardiomyoblasts with an overactive NHE1. Our results demonstrate that pro-Cat B protein levels were significantly elevated in the extracellular compartment (Figure 3.14). This effect was significantly regressed by inhibition of NHE1 or Cat B (Figure 3.14). Surprisingly, only pro Cat B levels were detected in the extracellular compartment. It is to be noted that Cat B pro form is indicated to be enzymatically inactive and requires an acidic pH to become active. Our *in vitro* model contain buffers in the culture media to maintain the pH of the cardiomyoblasts. Due to the presence of the buffers in our culture media we do no encounter an acidic extracellular environment which is required for Cat B activation. However, it has been reported that Cat B can retain significant proteolytic activity at neutral pH as well (88), and as such is capable of activating pro MMP-9 in the extracellular

environment (218). Once into the extracellular compartment, Cat B can cause further ECM degradation by activating MMP-9 (217, 218).

4.7 Increase in Cat B Protein in the Extracellular Compartment Induces an Increase in MMP-9 Activity

A recent study has demonstrated that acidic extracellular pH values increase MMP-9 and Cat B secretion (133). Cat B has also been shown to mediate the pH dependent activation of pro-MMP-9 (218). Moreover, Cat B and MMP-9 were shown to directly interact with NHE1 and cause ECM degradation (133). Hence, we aimed to determine the MMP-9 gelatinolytic activity in the conditioned media obtained from H9c2 cardiomyoblasts with overactive NHE1. Gelatin zymography revealed that MMP-9 gelatinolytic activity was significantly elevated following stimulation of NHE1 with Ang II (Figure 3.15). This effect was significantly regressed upon inhibition of NHE1 or Cat B (Figure 3.15). In our study, the gelatin zymograms were able to detect MMP-9 at 92 kDa only, which is in accordance with what has been observed in earlier studies (218, 253). The band corresponding to 92 kDa is mostly described as the inactive form of MMP-9. However, certain protein substrates, such as collagen IV and glycoproteins, have been shown to cause conformational changes within the MMP-9 structure that result in the exposure of the active site without cleavage of the pro-peptides (254, 255). MMP-9 activity has been shown to be increased in various models of heart failure (43, 219). Hence, activation of MMP-9 can result in ECM degradation, which can contribute further towards cardiomyocyte hypertrophy (43, 165, 219). We also looked at the MMP-9 protein

expression in the extracellular compartment. However, Immunoblot analysis revealed no change in MMP-9 protein expression in the conditioned media (Figure 3.16). It is to be noted that zymography is known to be a much more sensitive method than Western blotting as it is able to detect small amounts of activity as well. This might explain why our zymography experiments revealed changes in MMP-9 activity, whereas the immunoblot analysis did not.

4.8 NHE1 Induced Increase in Cat B Expression does not Activate Apoptosis

Cardiomyocyte apoptosis is commonly seen in the progression of heart failure (113, 114). Apoptosis is a cell death mechanism primarily regulated by caspases, a family of 14 cysteine proteases that cleave their substrates specifically at an Asp residue (115). Both extrinsic (death receptor-mediated) and intrinsic (mitochondrial- mediated) apoptotic pathways activate caspases which subsequently degrade numerous polypeptides in the cell including major structural elements, DNA repair machinery, and protein kinases (115). In cardiomyocytes, the predominant apoptotic pathway is intrinsic (116), primarily through Bcl-2 protein involvement, and procaspase activation (117, 118).

Cat B and NHE1 have independently been shown to be involved in apoptosis (123, 124) (244, 245). Inhibition of NHE1 activity via the specific inhibitor cariporide resulted in decreased apoptosis in isolated cardiomyocytes (256). Another study reported that in an ischemic rat model, the NHE1 inhibitor cariporide reduced apoptosis and this was associated with a significantly higher ratio of (antiapoptotic) Bcl-2 to (pro-apoptotic) Bax

genes (250, 257, 258). NHE1 has been implicated in NHE1 induced apoptosis in the myocardium (244, 245). Previous studies have demonstrated that cathepsins are essential for cardiomyocyte apoptosis (97) (123, 124). An absence of Cat B and L has been shown to induce neuronal loss and brain atrophy (125). Cat B has also been shown to mediate caspase-independent cell death in non-small cell lung cancer cells (126). Once Cat B is released from the lysosomes into the cytoplasm it may activate or enhance apoptotic pathways (126). Moreover, the antiapoptotic molecules Bcl-2, Bcl-xL, Mcl-1, and XLAP (X-chromosome linked inhibitor of apoptosis) are targeted by the lysosomal Cat B and L in several human cancer cell lines (127). Recently, Wu *et al* have demonstrated an increase in the apoptotic marker Bax mRNA and a decrease in the anti-apoptotic marker Bcl-2 mRNA in H9c2 cardiomyoblasts treated with Ang II (216). Hence, we wanted to see the effect of our treatment on the apoptotic markers, Bcl-2 and Bax mRNA. Our results revealed no changes in Bax and Bcl-2 mRNA (Figure 3.18 and 3.19). However, recently Wu *et al* demonstrated an increase in Bax protein expression and a decrease in Bcl-2 protein expression in H9c2 cardiomyoblasts upon stimulation with Ang II (216). The differences in our study and Wu *et al*'s may be due to the different concentrations of Ang II used in both studies (216). Based on our findings, we speculate that the cardiomyocytes might be undergoing a biphasic response in relation to autophagy and apoptosis, where one pathway may dominate the other one. Further studies are needed to better understand the crosstalk between autophagy and apoptosis in NHE1 induced CH. Our study showed that the Cat B mediated hypertrophic response induced the activation of the autophagic pathway alone without any activation in apoptosis.

CONCLUSION

Our study demonstrated for the first time that stimulation of NHE1 caused an increase in the Cat B protein and gene expression. This increase was regressed upon inhibition of NHE1 or Cat B (Figure 4.1). Moreover, cardiomyoblasts expressing active NHE1 significantly increased the cardiomyocyte hypertrophic markers (Figure 4.1). The inhibition of NHE1 or Cat B regressed this hypertrophic response indicating an important role of NHE1 in the Cat B induced cardiomyocyte hypertrophic pathway. Furthermore, stimulation of NHE1 caused the disruption of the lysosomes within the H9c2 cardiomyoblasts and also induced an increase in the LC3 protein levels. This indicates that the autophagy-lysosomal pathway plays a role in mediating the Cat B induced cardiomyocyte hypertrophy. The activation of the autophagy-lysosomal pathway caused the secretion of Cat B into the extracellular compartment where it activated pro-MMP-9. These effects seemed to be regressed upon inhibition of NHE1 or Cat B (Figure 4.1). The extent to which the conclusions of the present work may be applicable to native cardiomyoblasts and *in vivo* heart will require further studies using appropriate animal models. This shall confirm the results obtained in the present study.

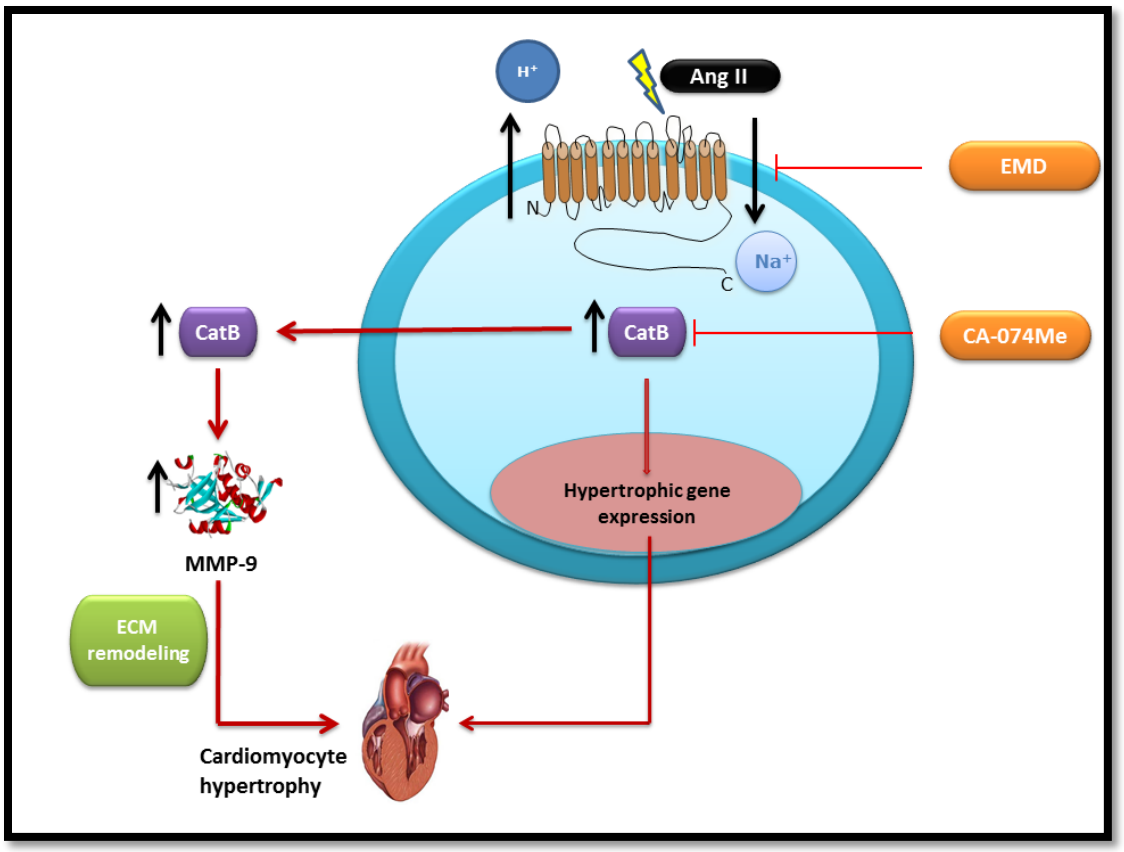


Figure 4.1 Cathepsin B and MMP-9 mediate NHE1-induced cardiomyocyte-hypertrophic response. The stimulation of NHE1 through Ang II causes an up regulation in Cat B protein and gene expression intracellularly. Cat B then moves into the extracellular compartment and activates MMP-9, resulting in the NHE1-induced cardiomyocyte hypertrophic response. NHE1, Na+/H+ Exchanger 1; Cat B, cathepsin B; MMP-9, matrix metalloproteinase-9

Chapter 6: FUTURE DIRECTIONS

5.1 Activation of NHE1 Causes ECM Degradation That is Mediated through Cat B and MMP-9

ECM consists of protein such as collagen and elastin which are vital for maintaining the integrity of the ECM network. Various isoforms of cathepsins and MMPs are able to degrade many ECM components such as fibrin, collagen and its isoforms, elastin. It has been reported that the elastolytic and collagenolytic activities in the LV myocardium of hypertensive heart failure rats were increased significantly in response to increased expression of in cathepsin S, K, L and B expression (36). Moreover, the elastolytic activity was significantly decreased upon treatment with E64, a broad spectrum cathepsin inhibitor and the collagenolytic activity was significantly decreased upon using GM6001, a MMP-2/9 inhibitor (36). Furthermore, NHE1 has been shown to directly interact with Cat B and MMP-9 and cause ECM degradation in a breast cancer cell line (133). The results of our study demonstrate that the protein levels of Cat B and MMP-9 activity was increased in the media. However, we did not investigate the effect of increased Cat B expression and MMP-9 activity upon their ECM substrates like elastin, and collagen. The logical next step would be to investigate the effect of overexpression of Cat B and MMP-9 in an *in vitro* and *in vivo* model upon elastin and collagen. In an *in vitro* model, this could be done by running elastolytic and collagenolytic assay in the absence or presence of Cat B or MMP-9 inhibitors. In an *in vivo* model, we can analyze the elastin and collagen compositions by performing IHC analysis and comparing with control hearts. For this we could utilize transgenic mice overexpressing the NHE1 (K-line). The results from these experiments

would provide us with conclusive evidence regarding ECM degradation due to NHE1 induced CH. This would provide conclusive evidence that the NHE1 induced increase in Cat B and in turn increase in MMP-9 activity is in fact mediating ECM degradation that contributes towards cardiomyocyte or CH.

5.2 Cat B Induced Cardiomyocyte Hypertrophy is Mediated through NHE1 and NF-kB

Nuclear factor-kappa B (NF-κB) is a ubiquitously expressed cellular transcription factor. NF-κB can be activated by various cytokines, such as tumor necrosis factor-alpha (TNF-α). Phosphorylation and degradation of I-κB alpha unit is necessary for its activation. NF-κB has been implicated in the regulation of cell survival, inflammation and immune cell maturation in cardiac myocytes. Several clinical studies have found a strong link between NF-κB activation and heart failure. It is suggested that NF-κB promotes heart failure by the activation of cytokines such as TNF-α and interleukin-1β (IL-1β). It has been demonstrated that failed human hearts contain activated forms of NF-κB, whereas normal hearts do not (259, 260). Moreover, it has been shown that polymorphisms in the promoter of the NF-κB gene causes the up regulation of NF-κB . Up regulation of NF-κB has been identified as a risk factor for DCM (261). A study of patients that received left ventricular assist devices demonstrated that reverse remodeling and enhanced function was associated with a decline in cardiac NF-κB activity (262). In cultured cardiac myocytes, hypertrophic agonists that activate $G_{\alpha q}$ coupled receptors, such as Ang II, ET-1, and phenylephrine, all promote nuclear translocation and transcriptional activity of NF-κB (263, 264). In addition,

NF-κB activity is required for the hypertrophic phenotype induced by these agonists and for the increased expression of atrial natriuretic factor (ANF) in postnatal ventricular cells, an important marker of fetal gene activation and pathological remodeling. *In vivo* studies have confirmed *in vitro* results, in that cardiac-specific expression of a mutant NF-κB IκBα subunit super-repressor attenuates the hypertrophic cardiac phenotype induced by Ang II and isoproterenol infusion, or low-grade aortic banding; as well as, the induction of a subset of fetal cardiac genes (265, 266). Furthermore, the NF-κB p50 subunit deficient mice display reduced CH following MI or during TNFα-induced cardiomyopathy (267, 268).

Interestingly, NHE blockade inhibits IL-1β and NF-κB in immunostimulated endothelial cells (269). Moreover, NF-κB has been shown to upregulate Cat B expression in H9c2 cardiomyoblasts (238). A potential NF-κB binding site could be identified on the gene promoter region of Cat B. It was also demonstrated that pretreatment of HeLa cells with specific NF-κB inhibitors abrogated the induction of Cat B expression (238). Up regulation of Cat B by NF-κB, followed by its secretion into the extracellular environment was seen in another cancer cell line (270). It was also demonstrated that the down-regulation of the NF-κB p65 subunit led to a reduction in Cat B expression in the same cell line (270). Taken together, it is clear that NF-κB is involved in CH and heart failure, but further studies are required to establish whether it function in cooperation with NHE1 or Cat B to promote CH or not. In order to further investigate the signaling pathways, isolated cells *in vitro* and lineage-specific knockouts should now be carried out to define the transcriptional targets and function of NF-κB in cardiomyocytes. For an *in vitro* model, H9c2 cell lines treated with prohypertrophic agents such as Ang II could be used to

stimulate NHE1. After treatment and incubation for a specified time, the nuclear fraction of H9c2 cells could be examined for NF-kB levels and NF-k B activity. Use of NF-kB inhibitors can be used to further tweeze out the signaling pathway and to determine whether NF-kB is in fact involved in the NHE1/CatB induced cardiomyocyte hypertrophic response.

5.3 Cat B Induced Cardiomyocyte Hypertrophy is Mediated through NHE1 and CD44

The CD44 is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. The CD44 is responsible for transducing intracellular signals in the myocardial cells. CD44 interact with various components of the ECM such as hyaluronan (HA), collagen, laminin, fibronectin and OPN (56). In MI hearts, CD44 is upregulated in fibroblasts, leukocytes, and endothelial cells (58). CD44 deficient mice subjected to MI show increased myocardial infiltration by leukocytes and expression of proinflammatory cytokines, followed by a decrease in fibroblast infiltration and fibrosis and enhanced dilative cardiac remodeling (58). Interestingly, a study demonstrated that the binding of HA to CD44 activates NHE1 activity which, in turn, creates an acidic extracellular matrix environment. The acidic extracellular environment leads Cat B activation. Increased Cat B activation causes degradation of the ECM which results in tumor progression. Moreover, inhibition of NHE1 activity by amiloride blocked NHE1 activity, reduced the acidic environment, and inhibited Cat B activation, and tumor cell invasion. Similar effects were seen upon downregulation of CD44 by using short interfering RNA (132). Therefore, it

would be interesting to investigate the effects of CD44 up regulation and inhibition on cardiomyocyte hypertrophic response. The results could further tweeze out the signaling pathways that lead to ECM remodeling in the NHE1/Cat B induced cardiomyocyte hypertrophy.

5.4 Cat B Induced Cardiomyocyte Hypertrophy is Mediated through NHE1 and IL-1β

IL-1β signaling is thought to be a vital mediator in the pathogenesis of cardiac remodeling. It has been demonstrated that the exogenous administration, *in vivo* and *in vitro,* has led to structural remodeling with reduced cardiac function (271). It has been suggested that IL-1β may induce systolic dysfunction in patients with heart failure, further supporting a negative role for this cytokine in heart disease (272, 273). Another study demonstrated a significant increase in the serum levels of IL-1β in rats after left anterior descending coronary artery ligation procedure compared with the sham control rats (96). In post-MI clinical trials, administration of IL-1 antagonist reduced adverse remodeling and improved cardiac function (274). Interestingly, IL-1β also increased the amounts of Cat K, B, and L mRNAs in cultured neonatal rat cardiomyocytes (36). In addition, previous studies have shown that Cat B and IL-1β work together to promote cardiac remodeling following MI in mice. (275). Evidence also indicates that NHEs are activated in response to IL-1β (276). Taken together, the mentioned reports suggests that IL-1β might be mediating the CH effects seen upon up regulation of NHE1 or Cat B. We hypothesize that increase in IL-1β levels cause an increase in NHE1 expression or activity which leads to

the increase in Cat B leading to CH. The hypothesis could be tested out in an *in vitro* model, by exposing the cardiomyocytes to IL-1β and measuring the changes in NHE1 activity and Cat B protein expression to a control non treated group. This would confirm whether IL-1β is in fact involved in the NHE1/Cat B induced cardiomyocyte hypertrophic cascade or not.

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