GENETIC MOUSE MODELS REVEAL KEY PHYSIOLOGICAL FUNCTIONS OF ADRENOMEDULLIN SIGNALING

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

Chapel Hill
2008

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ABSTRACT

Ryan Thomas Dackor: Genetic Mouse Models Reveal Key Physiological Functions of Adrenomedullin Signaling

(Under the direction of Dr. Kathleen M. Caron)

Adrenomedullin (AM) is a potent 52-amino acid peptide vasodilator that is involved in a wide variety of physiological processes, including regulation of renal function, neurotransmission, apoptosis and growth. AM peptide levels are elevated in many cardiovascular conditions, including normal pregnancy, septic shock, hypertension, and renal failure. The multitude of conditions associated with elevated AM levels suggests that it serves to maintain physiological homeostasis during various stresses. AM exerts most of its biological functions by promoting increases in the intracellular messengers, cAMP and/or nitric oxide. Recent characterization of AM signaling has identified a unique mechanism of G-protein coupled receptor signaling, mediated by a class of single transmembrane proteins called receptor activity modifying proteins (RAMPs), that have been shown to dictate ligand binding specificity of the calcitonin receptor like receptor (CLR for protein, *Calcrl* for gene). To date, three RAMP proteins have been identified and it is the association of RAMP2 or 3 with CLR that designates an AM receptor.

My research has utilized several lines of gene targeted mice to determine the *in vivo* role of AM and its signaling components in various physiologic contexts. I

show here that mice lacking CLR suffer from extreme hydrops fetalis and die at mid-gestation with severe cardiovascular defects, including small overall heart sizes, thin vascular smooth muscle cell walls and defects in myocardial proliferation and apoptosis. To further examine the role of CLR in cardiac development and physiology, I crossed mice with a floxed *Calcrl* allele to two cardiomyocyte-specific Cre lines, α-MHC and cardiac troponin. These mice develop normally and are born at the expected Mendelian ratios. Additionally, echocardiography and histological examination revealed no significant differences in heart structure or function as late as 14 weeks of age. Cardiomyocyte-specific CLR knockouts and control littermates also responded similarly to cardiac challenge in two different disease models: transverse aortic constriction and angiotensin II infusion. A separate study revealed that RAMP2 and RAMP3 have distinct physiological functions from embryogenesis to old age, whereby genetic deletion of *Ramp2* results in embryonic lethality while deletion of *Ramp3* has no effect on embryonic development or survival. Finally, I used mice heterozygous for the *AM* gene to show that the AM peptide is required for the normal inflammatory response to LPS-induced septic shock. Collectively, the work presented here provides the first *in vivo* genetic characterization of several key genes involved in AM signaling during various physiological conditions.

ACKNOWLEDGEMENTS

I would like to thank:

My advisor, Kathleen Caron

The Caron Lab, past and present members:

Will Dunworth, Kim Fritz-Six, Carrie Gibbons, Greg Harris

Mahita Kadmiel, Manyu Li, Gleb Rosanov

My Committee Members

Nobuyo Maeda
Mark Majesky
Joan Taylor
Da-Zhi Wang

My Parents, Gary and Gloria Dackor

My Wife, Jenn
For all of her love, support and advice.

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LIST OF ABBREVIATIONS

Akt	protein kinase B
ALT	alanine aminotransferase
AM	adrenomedullin
AMBP-1	adrenomedullin binding protein-1
Ang II	angiotensin II
AST	aspartate aminotransferase
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
calcrl	calcitonin receptor-like receptor gene
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CLP	cecal ligation and puncture
CLR	calcitonin receptor-like receptor protein
CT	calcitonin
ELISA	enzyme-linked immunosorbent assay
ES cells	embryonic stem cells
FBS	fetal bovine serum
GPCR	G-protein coupled receptor
GSK-3	glycogen synthase kinase-3
H&E	hematoxylin and eosin

h*ramp*	human *receptor activity modifying protein*
HSV	herpes simplex virus
I.P.	intraperitoneal
IL-1β	interleukin-1 beta
IL-6	interleukin-6
K_D	dissociation constant
L1	name of plasmid used to clone receptor
LBP	LPS binding protein
L-NAME	N-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
NF-κB	nuclear factor-kappa B
NHERF	sodium/hydrogen exchange regulatory factor
NO	nitric oxide
NSF	*N*-ethylmaleimide-sensitive factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDZ	Post synaptic density protein, Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1)
PFA	paraformaldehyde
PGK	phosphoglycerate kinase
PTX	pentoxifylline
RAMP1, 2 and 3	receptor activity modifying protein 1, 2 and 3
RDC-1	name of clone 1 from dog thyroid cDNA library

RenTgMK	renin transgene Marilyn Kozak
RT-PCR	reverse transcription-polymerase chain reaction
STAT3	signal transducer and activator of transcription 3
TBST	tris buffered saline with Tween 20
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VIP/VPAC1	vasointestinal peptide/pituitary adenylate cyclase activating peptide
VSMC	vascular smooth muscle cells
WT	wild-type
αMHC	alpha myosin heavy chain

CHAPTER 1

INTRODUCTION

Adrenomedullin: A Multifunctional Peptide

Adrenomedullin (AM) is a highly conserved, 52-amino acid peptide vasodilator that was first isolated from human pheochromocytoma in 1993 (45). Kitamura *et al*. discovered the peptide based on its ability to stimulate cAMP production in human platelets and maintain potent hypotensive activity in the rat. In addition to its vasodilatory activity, AM is involved in a wide range of other physiological actions including bronchodilation (38), renal function (72), neurotransmission (77), growth and apoptosis (80). AM is produced and secreted by almost all mammalian tissues and cell types, but it is most highly expressed in cardiovascular tissues such as the heart (35), lungs (76) and vasculature (88, 90). Stimuli for AM synthesis and secretion include inflammatory cytokines such as TNF-α and IL-1β (89), angiotensin II (75), endothelin-1 (75), hypoxia (25) and oxidative stress (2). Results from our literature summary of over 60 publications highlights the important role AM plays in various diseases and physiological conditions, especially cardiovascular disease, hepatic and renal disease, normal pregnancy and sepsis, where AM plasma levels are elevated at least 2-fold **(Figure 1.1)**. The multitude of conditions associated with elevated AM levels suggests that it may serve as a protective response to primary cardiovascular stress. In Japan, the AM peptide is already being used as a beneficial hemodynamic regulator for patients suffering from congestive heart failure (58). Therefore, AM is quickly becoming recognized as a ubiquitously expressed, dynamic peptide that can impact on several normal and pathological conditions.

AM Receptors and Signal Transduction

Biochemical and pharmacological studies on AM signaling has led to the characterization of a unique mechanism of G-protein coupled receptor signaling. However, the identification of a *bona fide* AM receptor has been difficult. Three putative AM receptors have been identified and suggested to mediate AM signaling based on their ability to bind the peptide and trigger an increase in cyclic AMP (cAMP) levels following AM treatment. One of these receptors, known as L1, binds to AM with a K_D of 8.2 X 10^{-9} M and mediates a cAMP response when expressed in COS-7 cells treated with the peptide (40). It is co-expressed with AM in most tissues, but in neonatal cardiomyocytes it is expressed at significantly lower levels than the other two putative AM receptors (4). Another receptor, RDC-1 was originally identified as a calcitonin gene-related peptide (CGRP) receptor, but also binds to AM at a K_D of 1.9 X 10^{-7} M and mediates a cAMP response to AM in COS-7 cells (41). A third receptor, known as the calcitonin receptor-like receptor (CLR, protein; *Calcrl*, gene), was cloned by several groups (1, 21), but inconsistent binding, expression, and functional results with AM and/or CGRP peptides confounded its declaration as a unique AM receptor (1, 17, 18, 21, 67). Several studies also contested the roles of L1 and RDC-1 as AM receptors (37, 43, 50, 52).

These discrepancies were eventually resolved with the identification of a class of single transmembrane proteins called receptor activity modifying proteins (RAMPs) that have been shown to dictate ligand binding specificity of CLR (52). To date, three RAMP proteins have been identified. As shown in **Figure 1.2**, when RAMP1 associates with CLR, a calcitonin gene-related peptide (CGRP) receptor is produced. On the other hand, association of RAMP2 or RAMP3 with CLR results in

a receptor that is specific for the AM peptide. CLR and the RAMPs are expressed in a variety of cell types and it is the spatial and temporal expression patterns of the RAMP proteins that determine how a cell will respond to either CGRP or AM.

The biochemistry and pharmacology of the RAMP-receptor interaction have recently been extensively reviewed by Hay *et al.* (22). Although the three mammalian RAMPs vary greatly in sequence homology (<30%) and tissue distribution (52, 79), they share a similar molecular mass (160 amino acids) and structure, which consists of a long extracellular amino terminus, a single transmembrane domain, and a short cytoplasmic carboxy terminus. The RAMPs have a broader tissue distribution than AM and CLR, which likely reflects the fact that RAMPs can interact with other class II GPCRs such as calcitonin (11), vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide 1, glucagon, PTH 1 and 2 receptors (10), and a class III GPCR called calcium-sensing receptor (5). Large scale, tissue-specific analysis of the human and mouse transcriptomes reveals that RAMP2 is among the top 10 genes the expression pattern of which correlates with that of CLR, suggesting that most CLR is complexed with RAMP2 to form a functional AM receptor (19, 87).

The first *in vivo* genetic evidence that CLR serves as the primary AM receptor is reported in this thesis (15). Briefly, gene targeting methods were used to generate and characterize mice lacking expression of the *Calcrl* gene. These mice die at mid-gestation from hydrops fetalis (generalized edema) and cardiovascular defects including thin vascular smooth muscle cell walls, small overall heart sizes and defects in cardiac proliferation and apoptosis. Interestingly, these defects almost

identically “phenocopy” those of the previously reported AM knockout mice (6) and provide compelling evidence that CLR acts as a *bona fide* AM receptor during embryonic development.

In a similar fashion, the validation of RAMP2 as a required component for AM signaling was also demonstrated by our laboratory (14). Briefly, *Ramp2* knockout mice die at mid-gestation with generalized edema that is remarkably similar to that seen in *AM* and *Calcrl* knockouts. Taken together, these *in vivo* studies define the receptor components required for AM signaling. In a surprising contrast, genetic deletion of *Ramp3* did not phenocopy the *AM*, *Calcrl* and *Ramp2* knockout mice. As presented in this thesis, apart from lean body mass at old age, *Ramp3* knockout mice appear mostly indistinguishable from their littermates. Therefore, the importance of RAMP3 in AM-mediated signal transduction remains to be determined.

Regulation of Growth and Apoptosis by AM

AM has various effects on cell growth and apoptosis (80), depending on cell type and experimental conditions. Conflicting results have been reported on the role of AM in vascular smooth muscle cell (VSMC) proliferation. Kano *et al*. showed that AM inhibits serum-stimulated proliferation of rat VSMCs through a cAMP-dependent process. (39) Another study by Upton *et al*. reported significant growth inhibitory effects of AM in platelet-derived growth factor (PDGF)-stimulated pulmonary artery smooth muscle cells (99). On the other hand, serum-deprived VSMCs responded to AM by synthesizing DNA and increasing the rate of proliferation. These responses

were independent of cAMP activation but were mediated by activation of the p42/p44 mitogen-activated protein kinase pathway (31). *AM* knockout mice have thinner VSMC walls than their wild-type littermates, providing *in vivo* evidence that AM is required for normal VSMC growth (6). Additional work, presented in this thesis, shows that *Calcrl* knockout mice also have thin VSMC walls caused by reduced proliferation, further supporting the role of AM signaling in VSMC growth (15).

The endothelium is a primary source of AM (88) and several studies have shown that AM plays a protective, pro-survival, anti-apoptotic role in endothelial cells. A study by Shichiri *et al*. demonstrated an anti-apoptotic role for AM in serum-deprived rat endothelial cells, whereby AM treatment induced expression of the pro-survival protein, Max (81). Furthermore, inhibition of cAMP or the addition of other cAMP elevating agents such as prostaglandin I2 or forskolin had no effect on apoptosis, suggesting that the anti-apoptotic activity of AM in this model was independent of cAMP. Another group also observed a cAMP-independent role for AM in preventing apoptosis in cultured human umbilical vein endothelial cells. In their experiment, Sata *et al*. found that the anti-apoptotic effect of AM was abrogated by L-NAME, but not by guanylate cyclase inhibition, suggesting an NO-dependent, cGMP-independent mechanism (78). In addition to regulating apoptosis, AM has also been shown to stimulate proliferation of endothelial cells (55, 56); a process that is important in angiogenesis and vascular remodeling of injured blood vessels.

The effects of AM on cardiomyocyte growth and apoptosis have also been well documented. AM prevents cardiac hypertrophy by inhibiting protein synthesis in

cardiomyocytes (97). Tokudome *et al.* demonstrated that AM inhibits doxorubicin-induced apoptosis in cardiomyocytes via a cAMP dependent mechanism (94). Two other reports also showed that AM attenuates myocardial apoptosis following ischemia-reperfusion in the rat via activation of the Akt-GSK pathway and reduced caspase-3 activation (68, 111). Work presented here shows that *AM* and *Calcrl* knockout mice have smaller overall heart sizes with reduced proliferation and increased apoptosis, supporting a pro-survival effect of AM signaling in the heart.

AM in Cardiac Function

Several studies have focused on the role of AM in cardiac function. Because systemic infusion of AM reduces peripheral resistance (61) (29), it has been difficult to determine whether AM has a direct effect on cardiac function or if the effects are secondary to reduced afterload. One group examined the effects of AM on blood pressure and changes in diastolic and systolic function following long-term administration of angiotensin II (Ang II) or norepinephrine in rats. They showed that AM suppressed the increase in blood pressure and augmented the improvement of systolic function induced by Ang II, but did not affect the development of left ventricular hypertrophy or cardiac gene expression (51). Another study showed that intravenous administration of AM enhanced left ventricular myocardial contraction and improved left ventricular relaxation without increasing myocardial oxygen consumption in patients with left ventricular dysfunction due to myocardial infarction (57).

Whether or not AM directly effects myocardial contractility, which would also contribute to enhanced cardiac function, is still controversial. Ikenouchi *et al.* showed that AM reduces contractility in rabbit ventricular myocytes by stimulating NO production, thereby decreasing intracellular Ca^{2+} concentration through a cGMP-dependent mechanism (28). On the other hand, AM has been shown to have a positive inotropic effect on isolated perfused rat heart and isolated rat papillary muscle (27, 91). Szokodi *et al*. reported that AM enhances myocardial contractility by stimulating Ca^{2+} release from intracellular ryanodine- and thapsigargin-sensitive Ca^{2+} stores, activating protein kinase C and increasing Ca^{2+} influx through L-type Ca^{2+} channels (91). Other studies have shown no inotropic effect (85) or even a dual inotropic effect, due to a switch from G_s to G_i coupling of the receptor after prolonged (30 minutes) exposure to AM (54). These contradictory results may be due to different experimental conditions and assay readouts. Some studies base their results on developed tension in muscle, while others measure individual cell shortening and/or intracellular Ca^{2+} levels in isolated cells. In addition, model species, amount of AM peptide used for treatment and method of muscle stimulation have varied across studies. No genetic models have been used to test the direct role of AM signaling in cardiac function. This thesis addresses the issue by using control and cardiomyocyte-specific *Calcrl* knockout mice to determine the role of AM signaling in overall heart function and cardiomyocyte contractility.

AM in Heart Disease

As previously stated, AM peptide levels are significantly elevated in a multitude of diseases, including cardiovascular disease. In patients with congestive heart failure, the concentration of plasma AM directly correlates with disease severity (36, 42, 63). In fact, AM has been identified as an independent predictor of prognosis in these patients (73). Furthermore, AM levels can be used to determine which patients should receive β-adrenergic antagonists for treatment of ischemic left ventricular dysfunction, since subjects with high AM benefit more from such treatment (74). Although AM is produced by various tissues, several studies suggest that myocardial AM production is a primary source of high plasma AM in patients with heart failure. AM immunoreactivity was higher in myocardial tissue obtained from heart transplant recipients with severe heart failure than from healthy donors (36). Unlike healthy subjects, patients with heart failure have markedly higher AM concentrations in coronary sinus (which receives blood from the whole heart) than in aorta, suggesting that a significant source of circulating AM is produced in the myocardium (34). AM concentration in patients with left ventricular dysfunction is significantly higher in pericardial fluid than in plasma (92). Animal models of heart failure induced by pressure or volume overload show a significant increase in myocardial AM, CLR, RAMP2 and RAMP3 gene expression, suggesting that the entire AM signaling system is involved in regulating the response to cardiac stress (13, 62, 64, 95, 112).

Several mechanisms probably regulate the overproduction of AM in the stressed heart including mechanical stretch of cardiomyocytes (96), hypoxia (12, 60) and elevations in vasocontrictors such as angiotensin II (98) and endothelin-1 (53).

Up-regulation likely serves as a protective response to cardiac stress by reducing preload and afterload. It may also act as a positive inotropic factor to increase contractility and cardiac output of the failing heart. Additionally, AM inhibits myocardial remodeling by attenuating cardiomyocyte hypertrophy and reducing both fibroblast proliferation and extracellular matrix production (33, 66). Finally, AM reduces aldosterone production (7), which is also up-regulated in heart failure. Therefore, AM cooperates with other natriuretic peptides to counteract the effects of vasoconstricting and sodium-retaining mediators such as endothelin and renin-angiotensin-aldosterone. Beneficial hemodynamic and neurohormonal effects of *iv*-infused AM into humans with heart failure include increased cardiac output and natriuresis, reduction of blood pressure and left ventricular end diastolic pressure, and increased ejection fraction (58).

Animal Models to Study AM in Heart Disease

Since $AM^{-/-}$ mice are embryonic lethal (6, 83), researchers have had to focus their phenotyping efforts on the viable $AM^{+/-}$ mice as a genetic model to study the role of AM in cardiovascular physiology and disease. However, in a positive way heterozygous mice more accurately mimic the human population, since polymorphisms in the *AM* gene may lead to similar levels of expression (47).

$AM^{+/-}$ mice have been used to demonstrate protective effects on cardiac tissue following aortic banding and angiotensin II infusion (65, 66). In general, these studies, and work from our own laboratory, have shown that $AM^{+/-}$ animals suffered greater degrees of cardiac hypertrophy and fibrosis and had a greater loss of overall

cardiac function. Two other studies showed that AM gene delivery attenuates hypertension, cardiac remodeling and renal injury in two hypertensive rat models; the Dahl salt-sensitive hypertensive rat, and the deoxycorticosterone acetate-salt hypertensive rat (16, 114). To date, no one has focused on the cardiomyocyte-specific role of AM signaling in cardiac challenge. Work presented here addresses this issue by using cardiomyocyte-specific *Calcrl* knockouts in two models; aortic banding and angiotensin II infusion.

AM in Sepsis

Of all the pathological conditions in which plasma levels of AM are elevated, sepsis ranks the highest; a whole order of magnitude higher than all other conditions (20). Sepsis is defined as the clinical syndrome that results from a host's uncontrolled inflammatory response to infection by activation of the innate immune system (49). This self-perpetuating response involves a complex network of inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 that normally function to contain and eliminate infection. However, when this immune response progresses uncontrollably, the consequence is sepsis, which can ultimately lead to septic shock, resulting in multiple organ dysfunction, cardiovascular failure and death. In fact, multiple organ failure due to severe sepsis and septic shock continues to be the most common cause of death in intensive care units (44). It is estimated that more than 750,000 patients will develop severe sepsis and septic shock each year in North America, resulting in an overall mortality rate of

30-50% (46). On average, it is estimated to cost $22,100 per patient with a total annual cost of $17 billion nationally (3).

Sepsis is initiated by an invading organism which can either stimulate the host response directly, or through various components produced by the organism. To initiate the host's immune response, Gram-positive bacteria present peptidoglycan and lipotiechic acid, while endotoxin (lipopolysaccharide [LPS]) is the primary threat presented by Gram-negative bacteria. A host response mediated by Gram-negative bacteria begins when LPS binds to LPS binding protein (LBP) in the plasma. This LPS:LBP complex then binds to a cell membrane receptor protein, CD14. The CD14:LBP:LPS complex is recognized by Toll-like receptor 4 (TLR4) which serves as the signal-transducing receptor for the activation of NF-κB. The culmination of these signaling cascades stimulates a wide range of host immune responses, including the generation of complement, aggregation of platelets and the release of inflammatory cytokines in a number of cell types (104).

Undoubtedly, TNF-α has been the most studied cytokine shown to play a key role in the pathogenesis of sepsis. TNF-α recruits and activates macrophages, lymphocytes and neutrophils, and stimulates the release of other proinflammatory cytokines. The degree of elevated serum TNF-α in patients with sepsis correlates directly with the severity of infection and indirectly with survival rate. In addition, human volunteers show a systemic release of TNF-α soon after injection with endotoxin. When administered to experimental animals, TNF-α evokes a state of shock similar to that observed in septic shock. Furthermore, the administration of a TNF-α antibody provides protection against septic shock in animals treated with

endotoxin, suggesting that overproduction of this cytokine is principally responsible for susceptibility (104).

Sepsis and septic shock lead to a wide range of physiological consequences. Septic patients suffer respiratory complications that often lead to lung injury and pulmonary edema (23). Kidney failure, requiring dialysis, is also frequent (100). The liver also suffers alterations which can result in jaundice and changes in blood sugar concentrations that often require insulin treatments, even in non-diabetic patients (86). Septic patients also experience a biphasic hemodynamic response. Initially, an early hyperdynamic response develops, characterized by increased cardiac output and tissue perfusion, increased oxygen delivery and decreased vascular resistance. This is followed by a later hypodynamic phase, characterized by reduced cardiac output and tissue perfusion, decreased oxygen delivery and increased vascular resistance **(Table 1.1)** (48).

Administration of LPS into animals results in a dramatic elevation of plasma AM (8, 9, 84), with the small intestine serving as one of the primary sources of AM production in this response (116). AM induction by LPS is TLR4-dependent, as C3H HeJ mice which have a mutated form of TLR4, show no changes in AM expression following LPS exposure (113). Several factors influence AM peptide levels during sepsis including circulating levels of LPS (109), decrease in clearance, and enhanced production by multiple affected organs (24). Additionally, expression of neutral endopeptidases, the key enzymes involved in AM degradation, are down-regulated in some tissues during sepsis and this likely contributes to the observed elevations in AM peptide levels in septic subjects (32).

Many studies have used the cecal ligation and puncture (CLP) model to study the action of AM in sepsis (70, 71, 101, 103, 109, 117). Similar to what is seen in septic patients, animals with CLP experience a hyperdynamic cardiovascular response which takes place 2-10 hours after CLP, followed by a hypodynamic cardiovascular response which starts approximately 16 hours after CLP. Using this model, plasma AM is elevated as early as 2 hours after CLP (when the hyperdynamic phase begins) and progressively increases up to 30 hours thereafter (26). Further studies then focused on the possible role of AM in the initiation of the early hyperdynamic phase of sepsis. Intravenous infusion of AM in rats mimicked the hyperdynamic response observed in sepsis, while administration of anti-AM antibodies after CLP prevented this response from occurring, demonstrating that AM does indeed play a key role in initiating the hyperdynamic phase in sepsis (101). Other animal models have also supported the role of AM as a causative factor of the early hyperdynamic phase (105). It is therefore surprising that transition to the later hypodynamic phase occurs in the presence of high plasma AM levels. Wang *et al*. addressed this issue in a subsequent study and showed that responsiveness to AM in thoracic aorta rings was not altered at 5-10 hours after CLP, but is significantly reduced after 20 hours of CLP (102). It was therefore suggested that transition to the hypodynamic phase is due to reduced vascular responsiveness to AM in the later stages of sepsis. Therapeutic interventions that would serve to maintain vascular responsiveness to AM might prevent or delay the progression to the hypodynamic phase and ultimately attenuate the severity of organ damage associated with sepsis.

Why there is reduced vascular responsiveness to AM in sepsis is unclear, but there are a few possibilities. First, as AM levels are increased following the onset of sepsis, there is a concomitant reduction in the amount of adrenomedullin binding protein-1 (AMBP-1), a protein that associates with AM to confer full biological activity. Indeed, *in vitro* addition of AMBP-1 successfully restored vascular responsiveness to AM following CLP, suggesting that reduced AMBP-1 levels during sepsis could account for vascular AM hyporesponsiveness and the transition to the hypodynamic phase (115). Another study showed that co-administration of both AM and AMBP-1 reduced the 10-day mortality rate in CLP-treated animals from 57% to 7%, while treatment of either factor alone had no significant effect on survival (108). A second possible explanation for reduced AM vascular responsiveness lies within the concomitant regulation of AM receptors and RAMPs, in which a dynamic change in expression also occurs during sepsis **(Table 1.1)**. For example, Nagoshi *et al.* found that TNF-α significantly reduced the expression of *Calcrl*, *Ramp1* and *Ramp2* in cultured human coronary artery smooth muscle cells in a time and dose-dependent manner, thereby diminishing the AM-induced production of cAMP (59). Furthermore, Ono *et al*. examined the expression of *Calcrl* and *Ramp1*, *2*, and *3* in several tissues from LPS-induced septic mice. *Calcrl* and *Ramp2* expression were singnificantly decreased in lungs of septic mice, whereas *Ramp3* message levels were increased approximately 40 fold in lungs after 12 hours of LPS challenge (69). Using a polymicrobial model of sepsis, Ornan *et al*. also showed that *Ramp3* expression is elevated in lungs during the early hyperdynamic stage of sepsis, but not in the later hypodynamic phase (71). Thus, combining the loss of available

AMBP-1 and altered expression of AM receptors probably plays a major role in the reduced AM vascular responsiveness observed during the septic response.

Several publications have reported beneficial effects of exogenous AM in sepsis. One group showed that AM stabilizes endothelial barrier function and reduces endothelial hyperpermeability, thereby preventing the development of pulmonary edema and improving survival in a rat model of α-toxin-induced septic shock (93). Zhou *et al*. showed that administration of AM with AMBP-1 early after the onset of sepsis significantly attenuated vascular endothelial cell apoptosis (118). In transgenic mice overexpressing AM in their vasculature, LPS treatment induces less hemodynamic and inflammatory alterations, less liver damage and lower mortality rates than in control animals (82).

Several studies have also demonstrated an important role for AM in inflammatory cytokine regulation. AM suppresses IL-1β-induced TNF-α production in Swiss 3T3 cells (30). Wu *et al*. demonstrated that AM and AMBP-1 both down-regulated LPS-induced TNF-α secretion in a macrophage cell line and in rat Kupffer cells. As expected, this effect was significantly enhanced when AM and AMBP-1 were co-administered (107). In agreement with these results, co-administration of AM and AMBP-1 5 hours after CLP significantly reduced plasma TNF-α, IL-1β and IL-6 at 20 hours after the onset of sepsis (110). Interestingly, a separate study showed that AM is both anti-inflammatory and pro-inflammatory, as it down-regulated TNF-α, but up-regulated IL-1β and IL-6 secretion from LPS-stimulated macrophages (106). Discrepancies in these studies likely result from different experimental conditions such as cell type and dosage/time of treatments.

Although numerous studies have used animal models to study the role of administered or increased AM in septic animals, genetic studies to determine the consequences of reduced AM during sepsis have not yet been performed. This study reports the use of mice heterozygous for the *AM* gene to examine the role of AM in regulating the inflammatory response to an LPS-induced model of sepsis.

Research Presented in this Dissertation

The overall goals of this dissertation have been to use genetically engineered animal models to define the receptor components essential for AM signaling *in vivo* and to determine the roles of AM signaling in two of the most clinically relevant conditions; cardiovascular disease and sepsis. The work presented in chapter 2 describes the generation and characterization of *Calcrl* knockout mice. Because these mice die *in utero* at mid-gestation from various cardiovascular defects, the next logical step was to study the role of *Calcrl* in specific cell types. Therefore, chapter 3 describes the generation and characterization of mice lacking *Calcrl* expression specifically in cardiomyocytes, while maintaining normal levels of expression in other cells. Briefly, cardiomyocyte-specific *Calcrl* knockouts are born at the expected Mendelian ratios, survive into adulthood with no obvious defects and respond normally to cardiac stress induced by aortic constriction and angiotensin II infusion. Chapter 4 describes the generation and characterization of *Ramp2* and *Ramp3* knockout mice. While *Ramp3* knockouts are essentially normal, mice lacking *Ramp2* die at mid-gestation from severe generalized edema, similar to *AM* and *Calcrl* knockouts. Finally, Chapter 5 presents work from another project which

used *AM* heterozygous mice to study the effects of reduced AM in a model of LPS-induced septic shock. Overall, this thesis elucidates the role of key genes involved in AM signaling in various developmental, physiological and pathological settings.

Figure 1.1

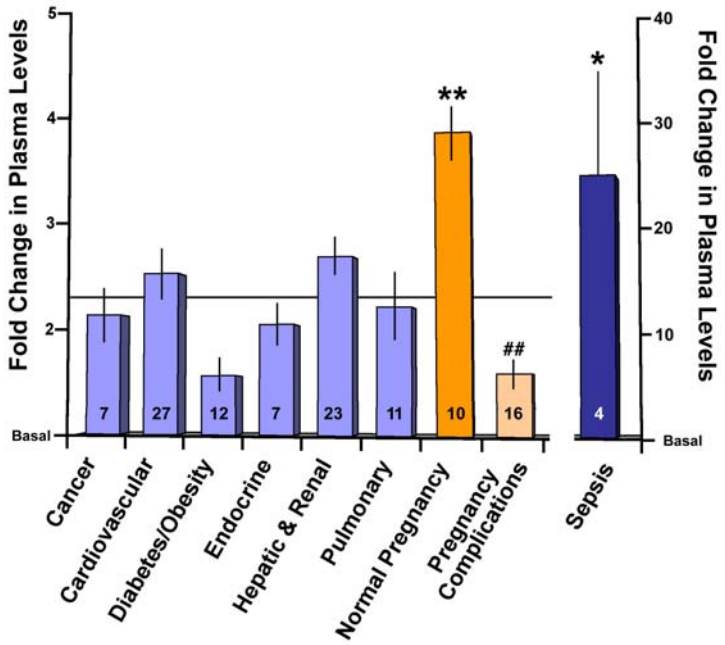

Fig. 1.1. Fold Change in Plasma AM Levels in a Variety of Human Conditions. *Bars* indicate average fold change in circulating AM levels in various disease categories or conditions based on published human clinical data. The *horizontal line* at 2.3 represents the average fold increase in plasma AM levels across all conditions (excluding sepsis). **, $P < 0.007$ between pregnancy and all other disease conditions (excluding sepsis). **##**, $P < 0.001$ between normal pregnancy and pregnancy complications. *, $P < 0.05$ between sepsis and all other conditions. *Number on each bar* indicates the number of published observations assessing plasma AM levels in each category.

Figure 1.2

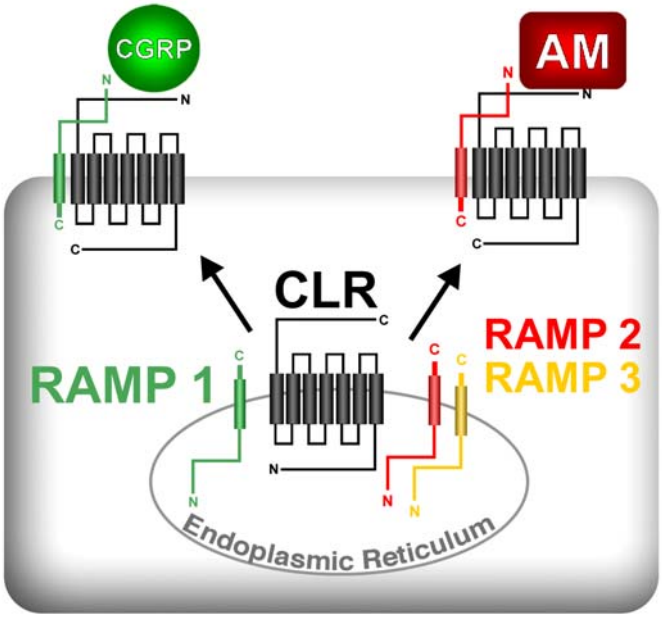

Fig. 1.2. The RAMP/Receptor Paradigm for AM and CGRP Signaling. RAMPs convey receptor specificity by heterodimer formation with CLR in the endoplasmic reticulum followed by localization to the plasma membrane. The association of CLR with a RAMP determines the specificity of ligand binding. Thus, a CLR/RAMP1 heterodimer (*green*) binds preferentially to CGRP, whereas association of CLR with either RAMP2 (*dark red*) or RAMP3 (*light yellow*) results in preferential binding to AM.

Table 1.1

The hyperdynamic and hypodynamic phases of sepsis

	Hyperdynamic (1-12 Hrs.)	Hypodynamic (12 Hrs. & later)
Cardiac Output	↑	↓
Tissue Perfusion	↑	↓
Oxygen Delivery	↑	↓
Vascular Resistance	↓	↑
Ramp2 Expression	Decreasing	↓↓
Ramp3 Expression	↑↑	Decreasing
Calcrl Expression	Decreasing	↓↓
Vascular Responsiveness to AM	Decreasing	↓↓

Table. 1.1. A biphasic hemodynamic response during sepsis (*Upper*). Initially, an early hyperdynamic response develops, characterized by increased cardiac output and tissue perfusion, increased oxygen delivery and decreased vascular resistance. This is followed by a later hypodynamic phase, characterized by reduced cardiac output and tissue perfusion, decreased oxygen delivery and increased vascular resistance. A dynamic expression pattern of AM receptor components during sepsis (*Lower*). During the first 12 hours of the septic response, *Ramp2* and *Calcrl* expression declines, while *Ramp3* expression is elevated. During the later hypodynamic phase of sepsis, *Ramp2* and *Calcrl* expression are reduced by ~95% and *Ramp3* expression begins to decrease.

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CHAPTER 2

HYDROPS FETALIS, CARDIOVASCULAR DEFECTS AND EMBRYONIC LETHALITY IN MICE LACKING THE *CALCITONIN RECEPTOR–LIKE RECEPTOR* GENE

Abstract

Adrenomedullin (AM) is a multi-functional peptide vasodilator that is essential for life. To date, numerous *in vitro* studies have suggested that AM can mediate its biological effects through at least three different receptors. To determine the *in vivo* importance of the most likely candidate receptor, calcitonin receptor like receptor (CLR), a gene targeted knockout model of the *Calcrl* gene was generated. Mice heterozygous for the targeted *Calcrl* allele appear normal, survive to adulthood and reproduce. However, heterozygote matings fail to produce viable *Calcrl*$^{-/-}$ pups, demonstrating that *Calcrl* is essential for survival. Timed matings confirmed that *Calcrl*$^{-/-}$ embryos die between day E13.5 and E14.5 of gestation. The *Calcrl*$^{-/-}$ embryos exhibit extreme hydrops fetalis and cardiovascular defects, including thin vascular smooth muscle walls and small, disorganized hearts remarkably similar to the previously characterized *AM*$^{-/-}$ phenotype. *In vivo* assays of cellular proliferation and apoptosis in the hearts and vasculature of *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ embryos support the concept that AM signaling is a crucial mediator of cardiovascular development. The *Calcrl* gene targeted mice provide the first *in vivo* genetic evidence that CLR functions as an AM receptor during embryonic development.

Introduction

Adrenomedullin (AM) is a potent peptide vasodilator that has been implicated in a wide variety of normal physiological processes including embryonic development (5), natriuresis (36), regulation of salt and water appetite (41, 48), cellular proliferation (7, 17, 49, 54), angiogenesis (11, 26) and anti-microbial defense (3). During many cardiovascular stresses such as pregnancy, septic shock, hypertension and renal failure, plasma levels of AM are dramatically elevated and thought to provide a protective homeostatic response, diminishing adverse tissue remodeling and fibrosis associated with cardiovascular stress (16, 35, 42-44).

Our previous studies using a genetically engineered *AM* knockout mouse model have demonstrated an essential role for the *AM* gene in the development of cardiovascular tissues (5). Mice lacking the *AM* gene suffer from extreme hydrops fetalis and die at mid-gestation. The most obvious phenotype of $AM^{-/-}$ embryos is severe interstitial fluid accumulation and generalized edema. Closer evaluation of the $AM^{-/-}$ embryos also revealed developmental cardiovascular defects that include thin vascular smooth muscle walls and smaller hearts with thin compact zones and disorganized trabeculae (5). However, the cellular mechanisms underlying these embryonic cardiovascular defects remain unclear.

Since the identification of the AM peptide over 10 years ago (27), three putative receptors have been identified and suggested to mediate the biological effects of AM based on their ability to bind the peptide and elicit a cAMP response to AM treatment *in vitro.* L1, originally cloned as an orphan receptor (10, 15), binds to AM with a Kd of 8.2×10^{-9} M and can mediate a cAMP response to AM when

expressed in COS-7 cells (21). It is co-expressed with the AM peptide in most tissues (21) but is found at significantly lower levels than the two other putative AM receptors in rat neonatal cardiac myocytes (4). A second receptor, RDC-1, was originally identified as a receptor for the AM-related peptide, calcitonin gene related peptide (CGRP), but also binds to AM with a Kd of 1.9 X 10^{-7} M and mediates a dose-dependant cAMP response to AM when expressed in COS-7 cells (22). A third receptor, commonly referred to as the calcitonin receptor-like receptor (CRLR), now usually referred to as the calcitonin-like receptor (CLR), was cloned independently by several groups (1, 14) but subsequently failed to produce consistent expression, binding, and functional results with CGRP or AM (1, 9, 12, 14, 45). Several recent studies have also failed to support the role of either L1 or RDC-1 as AM receptors (19, 25, 32, 39).

Most recently, the identification of a novel class of G-protein coupled receptor (GPCR) activity modifying proteins (RAMPs) and their association with CLR has helped to elucidate the most likely mechanism through which the AM peptide transduces its signal. Briefly, McLatchie *et al*. demonstrated that association with RAMP1 made CLR bind preferentially to CGRP while association of CLR with RAMP2 or RAMP3 made it bind to AM (39). This novel role of the RAMPs in GPCR cell signaling implies that the spatial and temporal expression of RAMP proteins dictates the presence and function of CLR as an AM receptor or a CGRP receptor and helps clarify some of the past confusion regarding AM signaling. However, no *in vivo* genetic studies to substantiate the identity of CLR as a functional AM receptor have been performed despite the great interest in the role of the peptide during

embryonic development and the possibility that modulating AM function might prove valuable for the treatment of cardiovascular disease.

In this paper, we used gene targeting in embryonic stem cells to generate and characterize mice that are deficient for the *Calcrl* gene, which encodes CLR. We find that although *Calcrl*$^{+/-}$ mice have no overt phenotypic defects, the *Calcrl* gene is essential for survival since no *Calcrl*$^{-/-}$ pups were ever born from heterozygote matings. Significantly, the embryonic lethal phenotype of the *Calcrl*$^{-/-}$ mice is remarkably similar to the phenotype we previously observed for *AM*$^{-/-}$ mice, including hydrops fetalis and developmental abnormalities in cardiovascular tissues (5). More detailed characterization of the growth properties of *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ vasculature and hearts has further confirmed an essential role for this signaling pathway in the growth and proliferation of the embryonic cardiovascular system.

Materials and Methods

Construction of the *Calcrl* Targeting Vector

To generate a *Calcrl* knockout targeting vector, a 129S6/SvEv genomic library was screened for phage clones containing the 5' portion of the *Calcrl* gene. A genomic clone consisting of approximately 11.5 kb and containing exons 3 through 9 of the *Calcrl* gene was used to isolate and clone a 5' short arm and 3' long arm of homology into a gene targeting vector (osdupdel) which contains multiple cloning sites flanking a PGK-neomycin cassette and also includes a HSV-thymidine kinase cassette. The 5.0 kb long arm of homology, which includes exons 7, 8 and 9, was isolated and subcloned using Hind III and Xho I restriction sites endogenous to the

gene locus. The 1.3 kb short arm of homology containing exon 4 was generated by PCR using the genomic phage clone as a template and oligo sequences that correspond to genomic sequences (5'-GGAAATTAGATTTTCAAGGGGTG-3' and 5'-GGCCTTTAAACTGTGAGCAAAG-3'). The short arm was inserted into the targeting vector by blunt ligation and the final targeting vector was linearized with Not I (Figure 2.1a).

Generation of *Calcrl*$^{+/-}$ ES Cells and *Calcrl*$^{-/-}$ mice

Standard gene targeting methods were utilized to generate ES cells and mice lacking CLR (30). Briefly, 129S6/SvEv-TC-1 embryonic stem cells were electroporated with the linearized targeting vector shown in Fig. 2.1a. After applying positive (G418) and negative (ganciclovir) selection, a positive ES cell clone was identified by PCR from over 800 selected clones. For PCR-based screening of targeted ES cells, we used 3 primers depicted in Fig. 2.1a; primer 1: 5'-GTGATTTGAGTCTGGAGA-3'; primer 3: 5'-GAAATGTGCTGTATGTTCAAG-3'; primer 4: 5'-TGGCGGACCGCTATCAGGAC-3'. Male chimeric mice that transmitted the targeted allele were bred to 129S6/SvEv females in order to establish an isogenic colony. To isolate *Calcrl*$^{-/-}$ embryos, heterozygote *Calcrl*$^{+/-}$ breedings were established and the day of the vaginal plug was considered embryonic day 0.5 (E0.5). For routine PCR-based genotyping of mice, we used a three primer strategy in which primer 2: 5'-GCTATGCTTTGTTTTCTGACA-3' and primer 3 amplify the wildtype allele while primer 2 and primer 4 amplify the targeted allele.

Generation of *AM*$^{-/-}$ mice

The generation, genotyping and characterization of mice with a targeted deletion of the *AM* gene have been previously been described (5).

Gene Expression Analysis

Calcrl gene expression was analyzed by real-time quantitative reverse transcription-PCR with the Mx3000P Real-Time PCR machine from Stratagene. Primers for *Calcrl* amplification were 5'-CAAGATCATGACGGCTCAATA-3' and 5'-CGTCATTCCAGCATAGCCAT-3'. The probe sequence for *Calcrl* detection was 5'-FAM-CATGCAGGACCCCATTCAACAAGCAT-TAMRA-3'. β-actin served as an internal control for all reactions. The primers used for β-actin amplification were 5'-CTGCCTGACGGCCAAGTC-3' and 5'CAAGAAGGAAGGCTGGAAAAGA-3'. The probe sequence for β-actin detection was 5'-TET-CACTATTGGCAACGAGCGGTTCCG-TAMRA-3'. RNA was isolated from E13.5 embryos with TRIzol Reagent (GIBCO/BRL) and subsequently DNase treated and purified with an RNeasy Mini Kit (Qiagen). 200 ng of total RNA was used in each reaction. The ΔΔCt method (33) was used to determine the relative levels of *Calcrl* expression and shown as a percentage of wild type. All assays were repeated three times, each with duplicates.

Histology

For histological analyses, embryos were dissected from the uterus at the desired stage of gestation, fixed in 4% paraformaldehyde (PFA), dehydrated, and

embedded in paraffin wax. 5 μm sections were mounted on slides for subsequent Hematoxylin & Eosin (H&E), anti-α-SMA, BrdU or TUNEL staining.

Anti-PECAM Staining

Tissues were fixed in 4% PFA in PBS overnight. Tissues were then cryoprotected with 30% sucrose in PBS overnight, embedded in OCT (Tissue-Tek) and cryosectioned at 10μm. Sections were rehydrated in PBS, quenched in 50mM NH_4Cl in PBS, permeabilized with 0.2% Triton/PBS and blocked in 3% BSA/1% FBS in PBS. Sections were incubated with anti-PECAM-1 (BD Pharmingen, cat# 550274) overnight at 4 degrees. After washing with TBST and PBS, sections were incubated with a Cy3 labeled donkey anti-rat secondary antibody (Jackson ImmunoResearch, code# 712-165-150) for 2 hours at room temperature. Sections were then washed with TBST and PBS and mounted for imaging. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera with Metamorph software (Molecular Devices Corp.) and processed in Photoshop.

Anti-SMαA Staining

Paraffin sections were deparaffinized and rehydrated and subsequently placed in 0.3% H_2O_2 in methanol for 15 minutes to block endogenous peroxidase activity. Sections were then rinsed in dH_2O and permeabilized in 3% BSA with 0.2% Triton x100 in PBS. After washing in PBS, specimens were incubated with anti-smooth muscle α actin (Sigma, cat# A2547) antibody for 1 hour at room temperature. Sections were then washed with TBST and PBS and incubated with an

HRP-labeled goat anti-mouse secondary antibody (Upstate, cat# 12-349) for 90 minutes at room temperature. After washing with TBST and PBS, peroxidase reaction was visualized with diaminobenzidine/hydrogen peroxide (Pierce, product# 34065), counterstained with 1% methyl green and mounted for imaging. Images were acquired on a Nikon FXA microscope and processed with Photoshop.

Cell Proliferation Assay

To label proliferating cells, pregnant mice received a single intraperitoneal injection of BrdU (Sigma B9285), using 100 mg BrdU per kg of body weight. One hour after injection, pregnant females were euthanized by cervical dislocation and embryos were prepared for histology as described above. BrdU was detected using the BrdU Staining kit from Zymed (#93-3943). Images were collected using a Leica MZ 16 FA dissecting microscope and the number of BrdU positive cells was quantified as the number of BrdU positive cells per area using Image J software.

TUNEL Cell Death Assay

Apoptotic cells were identified in 5 μm paraffin embedded sections using the ApopTag Fluorescein *In Situ* apoptosis detection kit (Chemicon) according to the manufacturer's protocol. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera with Metamorph software (Molecular Devices Corp.) and processed in Photoshop.

Statistics

Statistical analyses were performed with a Student's *t* test with unequal variance.

Experimental Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Results

Generation of mice lacking the *Calcrl* gene

Mice in which exons 5 and 6 of the *Calcrl* gene were deleted by homologous recombination were generated using the targeting strategy shown in Figure 2.1a. The disrupted allele, which lacks the *Calcrl* translation start site, was detected by Southern blot analysis using a genomic probe fragment located outside the areas of homology (Fig. 2.1b) and by PCR (Fig. 2.1c). To confirm that the gene targeting effectively disrupted transcription of the *Calcrl* gene, quantitative reverse transcription-PCR for *Calcrl* RNA was performed on total RNA isolated from whole embryos. As expected, *Calcrl*$^{+/-}$ embryos expressed approximately half of wildtype *Calcrl* RNA levels (38.2%, $p < 0.0001$ vs. wildtype) while *Calcrl*$^{-/-}$ embryos had no detectable levels of *Calcrl* RNA, thus confirming complete loss of *Calcrl* expression in knockout embryos (Fig. 2.1d).

Calcrl$^{-/-}$ mice die at mid-gestation with extreme hydrops fetalis

Mice heterozygous for the targeted *Calcrl* allele appeared normal at birth, survived to adulthood and reproduced. However, breeding *Calcrl*$^{+/-}$ mice failed to produce any viable *Calcrl*$^{-/-}$ offspring, demonstrating that the *Calcrl* gene is essential for survival. Timed matings between *Calcrl*$^{+/-}$ mice revealed that, although the homozygous null *Calcrl*$^{-/-}$ embryos were indistinguishable from their wildtype littermates at embryonic day 11.5 (E11.5, data not shown), by E12.5 the *Calcrl*$^{-/-}$ embryos were readily distinguishable from their *Calcrl*$^{+/+}$ and *Calcrl*$^{+/-}$ littermates by the presence of generalized, interstitial edema (Fig. 2.2a). The edema formation rapidly progressed so that by day E13.5 all *Calcrl*$^{-/-}$ embryos suffered from extreme hydrops fetalis (Fig. 2.2b) with an associated *in utero* mortality rate of approximately 50%. By E14.5 all *Calcrl*$^{-/-}$ embryos examined were dead. We note that our previous studies with *AM*$^{-/-}$ mice (5) revealed a similar type of generalized edema, however the onset of the edema occurred 24 hours later in gestation at E13.5.

Histological examination of *Calcrl*$^{-/-}$ embryos at E13.5 revealed distended skin due to fluid accumulation in the interstitial space, and a distended fluid-filled thoracic cavity (arrows in Fig. 2.2d, see also asterisk in Figs. 2.3e and 2.3f). We did not observe any significant hemorrhage in the hydropic *Calcrl*$^{-/-}$ embryos, suggesting that the blood vascular system remained structurally intact.

Calcrl$^{-/-}$ embryos have thin vascular smooth muscle walls

Numerous reports generated from *in vitro* experiments have shown that AM has either a negative or positive effect on vascular smooth muscle cell proliferation

(17, 20, 49, 55). Therefore, we used our *in vivo* genetic model to determine the role of AM signaling in the vascular smooth muscle cell layer of the developing aorta.

Histological comparison of the descending aorta revealed significantly fewer vascular smooth muscle cells in *Calcrl*$^{-/-}$ knockout embryos compared to their wildtype littermates at gestational days E12.5 and E13.5 (Figs 2.3b, 2.3c, 2.3e and 2.3f). This difference in vascular muscle wall thickness between *Calcrl*$^{-/-}$ and wildtype littermates was not apparent one day earlier in gestation at E11.5 (Figs 2.3a and 2.3d).

The percentage of BrdU positive cells in the vascular smooth muscle cell layer of the descending aorta of E12.5 embryos was quantified to determine the effects of CLR deletion on cellular proliferation (Fig. 2.3g). We found a significant reduction in the percentage of BrdU positive cells in the vessel walls of *Calcrl*$^{-/-}$ embryos when compared to wildtype littermates (17.67 +/- 1.17 for *Calcrl*$^{-/-}$ versus 27.64 +/- 2.01 for wildtype, $p \leq 0.005$).

To establish that the reduction in vascular smooth muscle wall thickness in *Calcrl*$^{-/-}$ embryos was not affected by abnormal smooth muscle cell differentiation or defects in endothelial patterning, we performed immunohistochemistry using antibodies against a smooth muscle marker, α-smooth muscle actin (α-SMA) and an endothelial marker, PECAM. As shown in Figures 2.3h and 2.3j, the aortic vascular smooth muscle cells of *Calcrl*$^{-/-}$ mice, like their wildtype counterparts, are positive for α-SMA, demonstrating normal vascular smooth muscle cell differentiation. Anti-PECAM staining also revealed a complete and well-formed endothelial lining in the aorta, demonstrating that endothelial tube formation and the final patterning of the

endothelial lining of the large vessels is unaffected in the major arteries of *Calcrl*$^{-/-}$ mice compared to wildtype embryos (Fig. 2.3i and 2.3k).

Calcrl$^{-/-}$ embryos have small and disorganized hearts

Transverse sections through the embryonic hearts at E11.5 revealed no obvious differences between *Calcrl*$^{-/-}$ and wildtype mice (Fig. 2.4a and 2.4d). However, by E12.5 (Fig. 2.4b and 2.4e) the *Calcrl*$^{-/-}$ embryos had significantly smaller hearts than their wildtype littermates. By E13.5 (Fig. 2.4c and 2.4f) the overall heart size of the *Calcrl*$^{-/-}$ embryonic heart was approximately two thirds the size of the wildtype littermate heart. The atria, mitral and tricuspid valves, endocardial cushion and ventricular septum appeared normal at all gestational ages (Fig. 2.4). Higher magnification of the left ventricle showed that at E12.5 and E13.5 the compact zone of *Calcrl*$^{-/-}$ hearts appeared thin and discontinuous (Fig 2.5 f-h) compared to wildtype controls (Fig 2.5 b-d). The myocardium also had a generally disorganized structure and the chamber appeared crowded (Fig. 2.5g and 2.5h). Immunohistochemical staining to characterize the presence and location of cardiomyocytes (α-actinin), endocardial cells (PECAM) and proliferating myofibroblasts (α- smooth muscle actin) revealed no obvious abnormalities in *Calcrl*$^{-/-}$ hearts compared to wildtype control hearts (data not shown).

Decreased cellular proliferation in *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ hearts

Based on our observation of smaller heart size and the fact that both AM and CGRP peptides have been previously shown to mediate cell growth, proliferation

and survival in a variety of different tissues (2, 17, 37, 57), we evaluated the extent of cell proliferation and apoptosis in developing *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ hearts at various gestational stages. Using the incorporation of BrdU as a measure of cell proliferation, we found no significant difference in the amount of proliferation in the ventricles of E11.5 *Calcrl*$^{-/-}$ embryos compared to wildtype littermates (101 +/- 3.25 for *Calcrl*$^{-/-}$ versus 101.6 +/- 4.14 for wildtype , p=0.927, Fig. 2.6a). In contrast, by E12.5 we found a significant reduction in the total number of BrdU positive cells in the ventricles of *Calcrl*$^{-/-}$ embryos compared to wildtype littermates (80.45 +/- 6.21 for *Calcrl*$^{-/-}$ versus 111.66 +/- 4.29 for wildtype, $p \leq 0.005$, Fig. 2.6a). Similarly, E13.5 *AM*$^{-/-}$ embryos showed a significant reduction in BrdU positive cells in the ventricles when compared to wildtype littermate controls (58.12 +/- 2.51 for *AM*$^{-/-}$ and 82.52 +/- 8.87 for wildtype, $p<0.05$, Fig. 2.6b).

Increased apoptosis in *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ hearts

Staining for apoptotic cells by TUNEL also revealed remarkable differences between the development of *Calcrl*$^{-/-}$ *and AM*$^{-/-}$ hearts compared to wildtype littermates. At E11.5 we found no obvious differences in the overall number of apoptotic cells which are normally present in the developing endocardial cushion, ventricular septum and ventricular apex of the heart (data not shown). Comparison of TUNEL staining in other organs of *Calcrl*$^{-/-}$ and wildtype littermates at E13.5 also revealed no significant difference in the number of apoptotic cells in the lung, dorsal root ganglia or the central canal of the spinal cord and only a slight increase in the liver (data not shown). However, by E13.5 we found a marked increase

(approximately 6 times more than wildtype) in the number of TUNEL-positive cells throughout the hearts, particularly in the ventricular apex, endocardial cushion and septum of *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ embryos compared to control littermates (Fig. 2.7).

Discussion

We used gene targeting in embryonic stem cells to generate and characterize mice that are deficient for the gene that encodes for one of the three putative AM receptors, CLR. Our most significant finding is that the *Calcrl* gene is essential for survival since *Calcrl*$^{-/-}$ mice die *in utero* at mid-gestation. Significantly, the embryonic lethal phenotype of the *Calcrl*$^{-/-}$ mice is almost indistinguishable from the phenotype we previously characterized for mice carrying a targeted deletion of the AM peptide (5). These shared phenotypes include severe generalized edema, developmental abnormalities in cardiovascular tissues that consist of reduced vascular smooth muscle cell development in the large arteries, and a small overall heart size with a thin and discontinuous compact zone. Although biochemical studies have identified at least three putative receptors for AM peptide signaling, the remarkable similarity between the phenotypes observed for these two knockout models provides compelling genetic and *in vivo* evidence that CLR is the primary receptor through which AM peptide acts during embryonic development. However, our results do not exclude the possibility that L1 and/or RDC1 also contribute to the function of AM at the same stage of life.

Another recent description of a gene targeted mouse model by Czyzyk *et al.* also provides additional compelling support for our conclusion that disruption of AM

signaling during embryonic development results in the consistent phenotype we describe. In their study, the authors show that deletion of the gene that encodes for peptidylglycine alpha-amidating monooxygenase (PAM), an enzyme that serves as the sole source of peptide amidation in the mouse, results in embryonic lethality, edema and cardiovascular defects that phenocopy the *AM* and *Calcrl* knockout models. Since amidation of AM peptide is required for its biological activity, the authors conclude that lack of amidation results in loss of AM function, presumably by reducing its ability to bind to its receptor(s), with a resulting phenotype that is strikingly similar to that seen in *AM* knockout embryos. Because several mouse models for deletion of genes that encode for other amidated peptides exist in which similar phenotypes are not observed, it is likely that inactivation of AM signaling in PAM mutants is the primary cause of the observed phenotype (8). Taken together, the description of a similar phenotype for three separate knockout mouse models demonstrates that abnormal cardiovascular development and generalized edema can be expected when AM signaling is disrupted during embryonic development.

The only significant difference we found between the CLR knockout and AM peptide knockout models is the timing of phenotypic onset: the edema and cardiovascular defects appeared 24 hours earlier in the *Calcrl*$^{-/-}$ knockout embryos (E12.5) than in the *AM*$^{-/-}$ knockout embryos (E13.5) (5). Given the recent finding that RAMP proteins determine the ligand binding affinity for CLR to either the CGRP peptide or the AM peptide, the most likely explanation for the difference in time of phenotypic onset is that *Calcrl*$^{-/-}$ mice have lost the ability to transduce signal for both AM and CGRP peptides by virtue of losing a shared GPCR. The *AM* knockout mice

may survive a while longer because they still have CGRP peptide signaling which is probably absent in the more severely affected *Calcrl*$^{-/-}$ mice. However, it is clear that CGRP signaling is not essential for survival since CGRP peptide knockout mice develop normally and survive to adulthood with only modest defects in blood pressure regulation and sympathetic nervous activity (13, 34, 47, 58). In addition, our experiments do not rule out the possibility that CLR, perhaps in association with different RAMP proteins, may bind and mediate the function of other unidentified peptide ligands. Thus, the precise reason for the difference in gestational phenotypic onset between the *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ mice remains an ongoing area of investigation.

The cause of edema in the *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ mice also requires further investigation. Embryonic lethality due to cardiovascular defects in genetically engineered murine models is sometimes associated with embryonic edema (6, 31, 38, 46, 50). However, the edema is usually mild, localized to the subcutaneous region and accompanied by hemorrhage and/or a blood-filled liver. In contrast, two recent reports demonstrate that generalized, interstitial edema similar to that seen in the *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ knockouts is caused by abnormalities in or failure of lymphatic vessel development (23, 56). Given the role of AM as an angiogenic factor, it is possible that a lack of AM signaling by genetic deletion of either the AM peptide or the CLR receptor results in lymphatic defects that cause severe and generalized hydrops fetalis. Current data is not yet sufficient to conclude the cause of edema in our mice.

It is well appreciated that AM peptide can differentially affect the growth properties of various cell types (2, 17, 37, 57). For example, while AM can inhibit apoptosis of cardiomyocytes (24, 51), it can also promote endothelial and vascular smooth muscle cell proliferation (17, 40). The effects of enhanced or reduced AM signaling on cells of the cardiovascular system are of particular interest since AM peptide levels dramatically increase in patients suffering from many cardiovascular conditions, including congestive heart failure (18), hypertension (29), myocardial infarction (28) and cardiac hypertrophy (52), and may provide protection against the development of adverse tissue remodeling and fibrosis associated with cardiovascular stress (43, 44, 53). Our data provide the first *in vivo*, genetic evidence that the *AM* and *Calcrl* genes allow transduction of essential signals during development that positively mediate the growth and proliferation of vascular smooth muscle cells and cardiac cells while concurrently negatively influencing cardiac cell apoptosis.

In conclusion, our studies using a genetically engineered knockout model for the *Calcrl* gene demonstrate an essential role for the CLR GPCR during embryonic cardiovascular development. The remarkable similarity between the *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ embryonic phenotypes leads us to conclude that CLR is the predominant receptor mediating AM signaling during development.

Figure 2.1

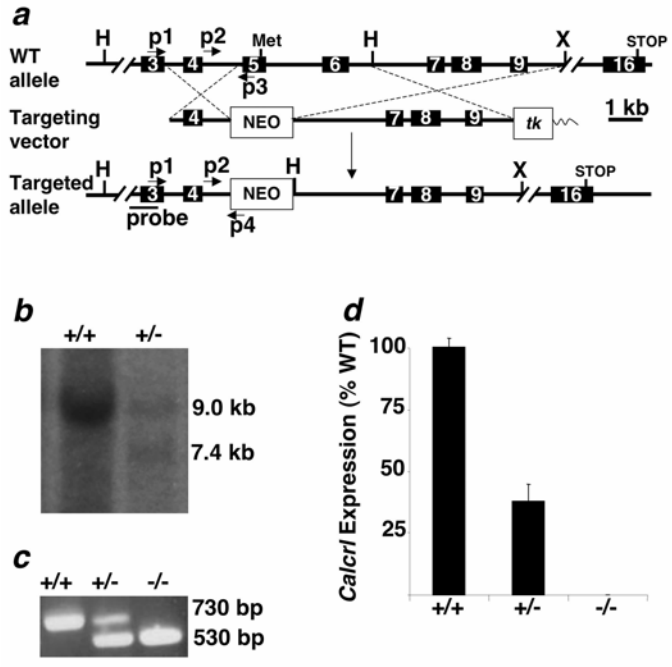

FIG. 2.1. **Generation of *Calcrl*$^{-/-}$ animals by homologous recombination**. (*a*) Strategy to disrupt the *Calcrl* gene. (*Top*) Endogenous wildtype allele. (*Middle*) Targeting vector. (*Bottom*) Targeted allele following homologous recombination. Primer locations for PCR (p1, p2, p3, p4) are shown with arrows. The location of the probe used for the Southern-based detection strategy is indicated by a labeled line (PROBE). The targeting vector plasmid sequence is indicated by a thin wavy line. Restriction sites: H, HindIII; X, XhoI. The initiator methionine and terminator codons are indicated as Met and STOP. (*b*) Detection of targeted ES cells by Southern blot analysis. Digestion of genomic DNA with HindIII results in a 9.0 kb fragment for the WT allele and a 7.4 kb fragment for the targeted allele when probed with the fragment depicted in *a*. (*c*) Primers depicted in *a* were used to amplify genomic DNA from embryos. (*d*) Measurement of *Calcrl* expression from total RNA extracts by real-time quantitative reverse transcription PCR. The relative quantity of *Calcrl* RNA in *Calcrl*$^{+/-}$ and *Calcrl*$^{-/-}$ embryos is represented as a percentage of total *Calcrl* RNA in WT embryos. Error bars represent SEM.

Figure 2.2

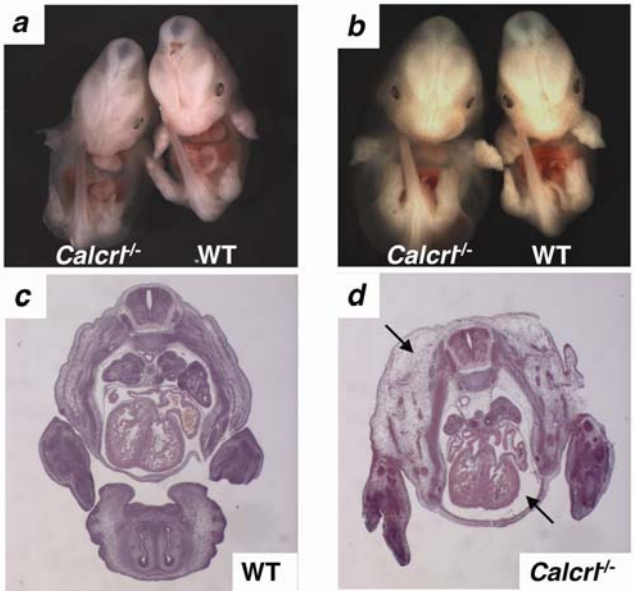

FIG. 2.2. *Calcrl*$^{-/-}$ embryos have massive generalized edema. (*a*) Generalized edema is observed throughout the entire body in *Calcrl*$^{-/-}$ embryos at E12.5. (*b*) By E13.5 the generalized edema in *Calcrl*$^{-/-}$ embryos has progressed to severe hydrops fetalis. (*c* and *d*) H&E stain of transverse sections through E13.5 WT (*c*) and *Calcrl*$^{-/-}$ (*d*) embryos. The thoracic cavity and interstitial tissues are filled with fluid and distended (see arrows in *d*, X1).

Figure 2.3

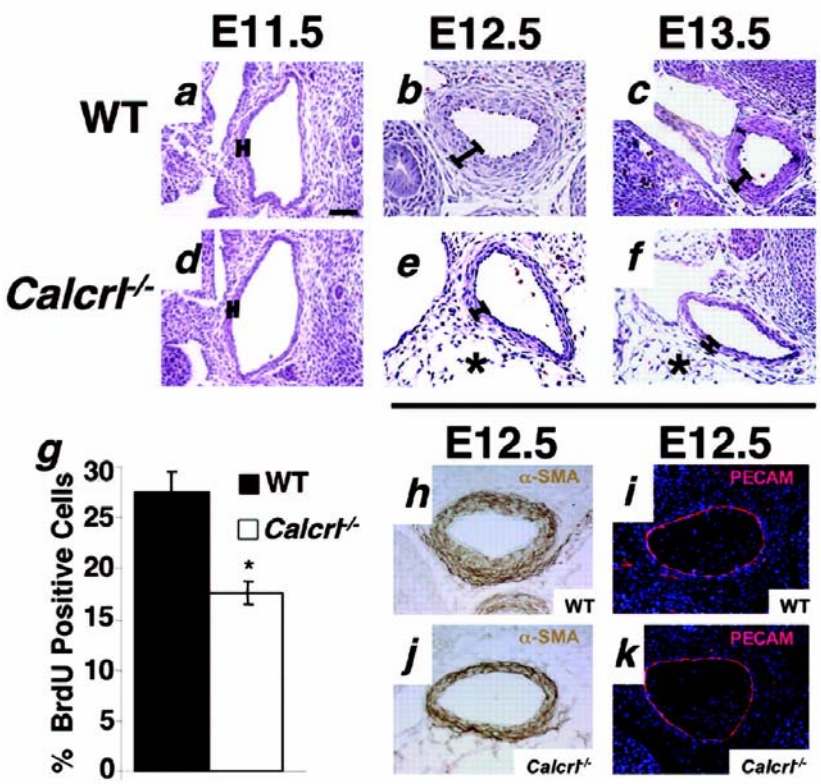

FIG. 2.3. ***Calcrl*$^{-/-}$ embryos have thin arterial walls due to reduction in vascular smooth muscle cell proliferation**. Transverse sections through the descending aorta of wildtype and *Calcrl*$^{-/-}$ embryos at E11.5, 12.5 and 13.5 were stained with H&E (a-f). The thickness of the vessel walls at E11.5 is similar in wildtype and *Calcrl*$^{-/-}$ sections. However, by E12.5 and E13.5 the vascular walls are thinner in *Calcrl*$^{-/-}$ mice (approximately 3 cells thick) when compared to wildtype controls (approximately 6 cells thick). The asterisk denotes the accumulation of interstitial edema in *Calcrl*$^{-/-}$ embryos. The percentage of proliferating, BrdU-positive cells in the aortic wall of *Calcrl*$^{-/-}$ mice is significantly less than in wildtype controls (g). Vascular smooth muscle cells in wildtype and *Calcrl*$^{-/-}$ aortas stain positive for α-SMA (h & j). Anti-PECAM staining shows that wildtype and *Calcrl*$^{-/-}$ aortas have normal endothelial patterning (i and k). a, aorta (X10). Scale bar, 50 μm.

Figure 2.4

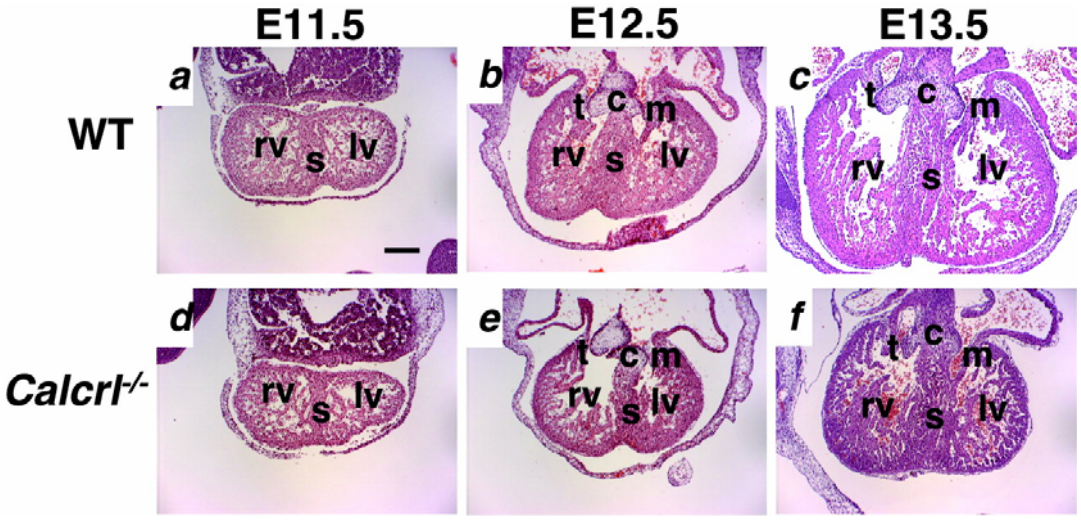

FIG. 2.4. *Calcrl*$^{-/-}$ embryos have developmental heart defects. Transverse sections through the hearts of wildtype and *Calcrl*$^{-/-}$ embryos at E11.5, 12.5 and 13.5 were stained with H&E. At E11.5 (a and d) the overall heart size is similar in both wildtype and *Calcrl*$^{-/-}$ embryos. At E12.5 (b and e), the overall heart size in the *Calcrl*$^{-/-}$ embryo is smaller than that of its wildtype littermate. At E13.5 (c and f), the overall heart size in the *Calcrl*$^{-/-}$ embryo is approximately two thirds the size of its wildtype littermate. c, endocardial cushion; s, septum; t, tricuspid valve; m, mitral valve; rv, right ventricle; lv, left ventricle; ch, chamber; cz, compact zone (X4). Scale bar, 200 µm.

Figure 2.5

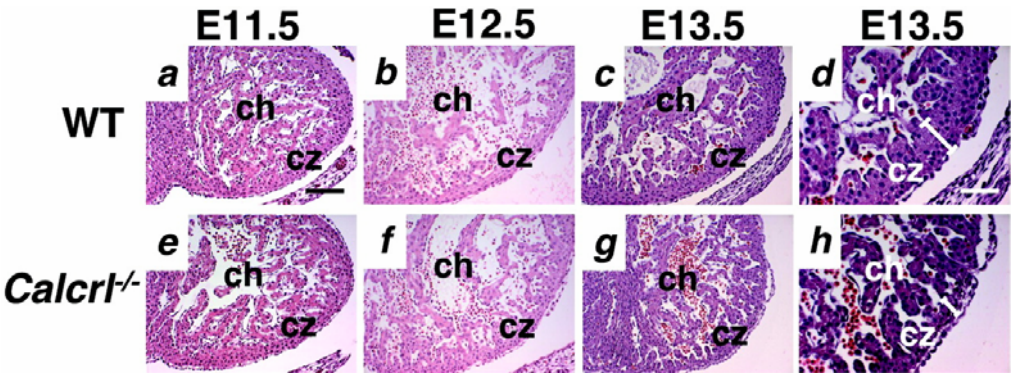

FIG. 2.5. *Calcrl*$^{-/-}$ embryos show thin and disorganized compact zones of the heart. Transverse sections through the hearts of wildtype and *Calcrl*$^{-/-}$ embryos at E11.5, 12.5 and 13.5 were stained with H&E. The compact zone in wildtype and *Calcrl*$^{-/-}$ embryos at E11.5 (a and e) and E12.5 (b and f) is similar in thickness and cellular organization. At E13.5 (c and g) the compact zone is thinner in the *Calcrl*$^{-/-}$ embryo. Higher magnification (d and h) reveals a discontinuous and convoluted organization of the compact zone in the *Calcrl*$^{-/-}$ section. ch, chamber; cz, compact zone (a, b, c, e, f, g X10 and d, g X20). Scale bar for X10 images is 100 μm; for X20 images scale bar is 50 μm.

Figure 2.6

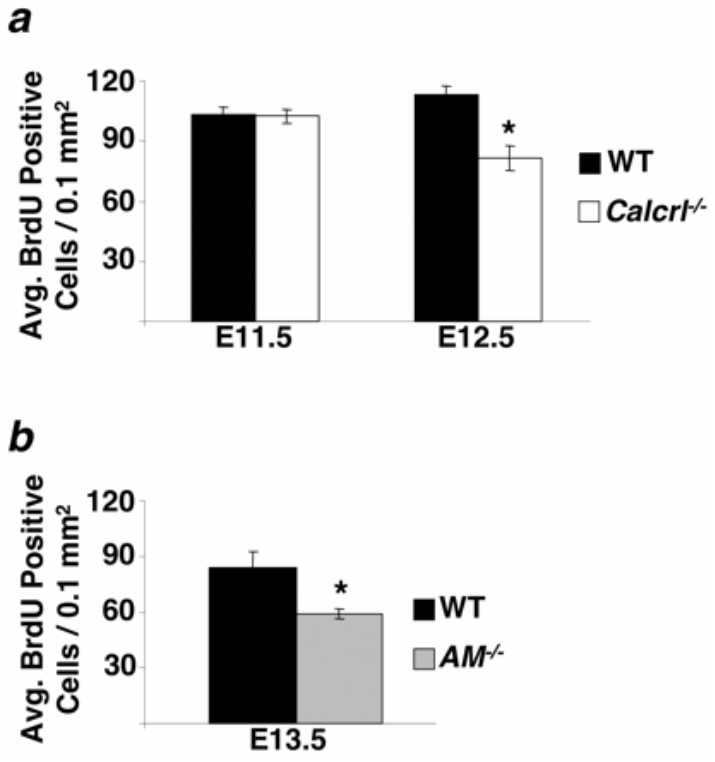

FIG. 2.6. *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ embryos have defects in cardiac cell proliferation. The proliferation index of cardiac cells was determined as the number of BrdU positive cells per total area in transverse heart sections. (*a*) No significant difference was found between wildtype and *Calcrl*$^{-/-}$ embryos at E11.5. However, at E12.5 the proliferation index was significantly lower in *Calcrl*$^{-/-}$ embryos when compared to wildtype controls. (*b*) The proliferation index of *AM*$^{-/-}$ embryos at E13.5 is significantly lower than wildtype littermates.

Figure 2.7

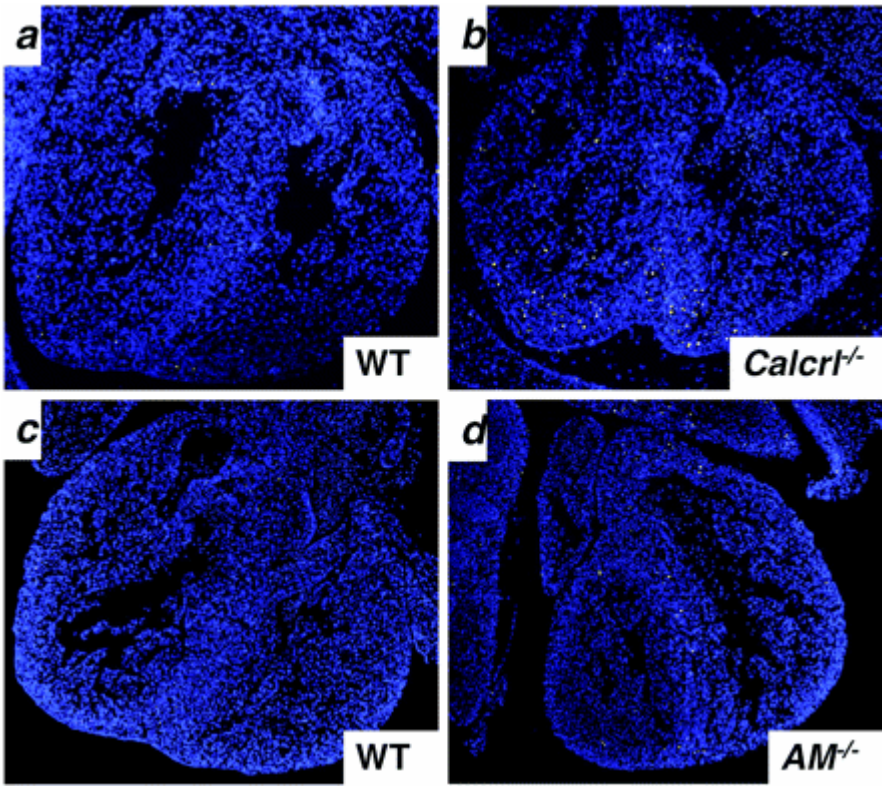

FIG. 2.7. *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ embryos at E13.5 have increased levels of cardiac apoptosis. Typical pictures of TUNEL stained transverse sections. (*a*) The number of TUNEL positive cells is greater in *Calcrl*$^{-/-}$ and *AM*$^{-/-}$ hearts (*b* and *d*, respectively) when compared to wildtype littermates (*a* and *c*).

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CHAPTER 3

CARDIOMYOCYTE-SPECIFIC DELETION OF THE *CALCITONIN RECEPTOR-LIKE RECEPTOR* (*CALCRL*) HAS NO EFFECT ON CARDIAC DEVELOPMENT OR FUNCTION

Abstract

Adrenomedullin (AM) is a dynamic regulatory peptide that is expressed and secreted by several cell types including vascular smooth muscle cells, endothelial cells and cardiomyocytes. AM signaling is mediated by a 7-transmembrane G-protein coupled receptor called the calcitonin receptor-like receptor (CLR, protein; *Calcrl*, gene). Multiple animal and human studies have focused on the role of AM in cardiovascular physiology and disease. The consistent observation is that AM serves a protective role in heart disease. However, our current understanding of AM physiology is incomplete due to several factors, including embryonic lethality of *AM* and *Calcrl* knockout mice and a broad expression pattern of *AM* and genes involved in its signaling. To enhance our understanding of AM signaling in cardiac physiology and disease, we deleted the *Calcrl* gene specifically in cardiomyocytes by crossing "floxed" *Calcrl* mice to mice expressing Cre-recombinase under the control of the alpha-myosin heavy chain promoter. Cardiomyocyte-specific *Calcrl* knockouts were born at the expected Mendelian ratios and had normal cardiac structure and function. The hypertrophic response to aortic constriction and angiotensin II infusion was also similar in controls and knockouts. Furthermore, cardiomyocyte contractility was not altered in knockout cells. Conclusively, this study shows that genetic deletion of the AM receptor, *Calcrl*, specifically in cardiomyocytes has no effect on cardiac development, function, or response to different disease models. It is thus likely that the cardioprotective effects of AM are mediated through another cell type, such as endothelial cells and/or vascular smooth muscle cells.

Introduction

Adrenomedullin (AM) is a dynamic peptide that is expressed and secreted from various tissues of the cardiovascular system including the kidneys, heart, lung and blood vessels (11). The receptor for AM, which is co-expressed in the same cells that secrete AM, is a G-protein coupled receptor called the calcitonin receptor-like receptor (*Calcrl*, gene; CLR, protein) (19, 25). AM peptide and the expression of its receptor are significantly elevated in several conditions such as normal pregnancy (10, 43), cardiovascular disease (17, 18, 22, 30, 31, 33, 45) and cancer (12, 40). It is therefore likely that AM is a key factor in regulating the physiological processes performed by the cardiovascular system in both normal and pathophysiological situations.

A number of reports, from both animal and human studies, have demonstrated a protective role for AM in heart disease. We recently demonstrated that mice with 50% expression of AM ($AM^{+/-}$) develop greater degrees of cardiac hypertrophy and renal damage in a genetic model of renin-induced hypertension (4). A study by Niu *et al*. also used $AM^{+/-}$ mice to show that endogenous AM is protective against cardiac hypertrophy and fibrosis induced by aortic constriction or Ang II infusion (35). Furthermore, intravenous infusion of AM into patients who had recently suffered a myocardial infarction revealed several beneficial effects including decreased left ventricular systolic pressure, increased myocardial contractility, increased cardiac index and improved left ventricular relaxation (27, 28).

There are many mechanisms by which AM may exert its cardioprotective effects. Because AM acts as a potent vasodilator, which ultimately reduces

peripheral resistance, many of its beneficial effects on the heart could be secondary to reduced afterload (23). However, several reports have also demonstrated direct effects of AM on cardiomyocyte function and growth. Whether or not AM directly affects myocardial contractility is still controversial. Some groups have observed a negative, positive or no inotropic effect on myocardial contractility (13, 14, 41, 42). One group even found a dual inotropic effect, due to a switch from G_s to G_i coupling of the receptor after prolonged (30 minutes) exposure to AM (26). These contradictory results may be due to different experimental conditions and assay readouts. Some studies base their results on developed tension in muscle, while others measure individual cell shortening and/or intracellular Ca^{2+} levels in isolated cells. In addition, model species, amount of AM peptide used for treatment and method of muscle stimulation have varied across studies.

The effects of AM on cardiomyocyte growth and apoptosis have been well documented. Previous reports from our lab show that mice lacking either the *AM* or *Calcrl* gene develop small hearts and have defects in myocardial proliferation and apoptosis (9). Tokudome *et al.* demonstrated that AM inhibits doxorubicin-induced apoptosis in cardiomyocytes via a cAMP dependent mechanism (44). Two other reports also showed that AM attenuates myocardial apoptosis following ischemia-reperfusion in the rat via activation of the Akt-GSK pathway and reduced caspase-3 activation (36, 47). Finally, AM prevents cardiac hypertrophy by inhibiting protein synthesis in cardiomyocytes (46).

Our previous studies using genetically engineered knockout mouse models for both the *AM* and *Calcrl* genes, demonstrated that AM signaling is essential for life

(5, 9). Mice lacking either gene suffer from extreme hydrops fetalis (generalized edema) due to abnormal development of the lymphatic vascular system (JCI in press). These mice eventually die at mid-gestation with multiple cardiovascular defects. Because these phenotypes are complex and likely interrelated, it becomes challenging to determine the cell autonomous cause of death in global knockouts. In addition, the gestational lethality of *AM* and *Calcrl* knockout mice precludes their use for investigating the potential cardioprotective mechanism of AM signaling in adult animals. Therefore, we have addressed the role of AM signaling in cardiac development and physiology by generating a mouse model that lacks *Calcrl* expression specifically in cardiomyocytes, while maintaining normal expression levels in other cell types.

Materials and Methods

Experimental Animals

The generation of *Calcrl*$^{Flox/Flox}$ mice has been previously reported (JCI in press). Cardiomyocyte-specific *Calcrl* null mice were generated by crossing *Calcrl*$^{+/-}$ mice to *αMHCCre*$^{+}$ mice (1). The resulting *Calcrl*$^{+/-}$;*αMHCCre*$^{+}$ animals were then bred to *Calcrl*$^{Flox/Flox}$ mice, resulting in the experimental animals used for this study. Experiments were performed with littermates of *Calcrl*$^{+/LoxP}$;*αMHCCre*$^{+}$ for controls and *Calcrl*$^{-/LoxP}$;*αMHCCre*$^{+}$ for knockouts. All mice used for this study were males and were between 12 to 15 weeks of age.

PCR detection of the *αMHCCre* transgene was performed using previously described primers (1). Genotyping for the *Calcrl* allele was performed with the same

three-primer strategy used for our original *Calcrl* gene targeted mice (9). The recombined *Calcrl* allele, produced by Cre-mediated recombination of the *Calcrl* floxed allele, was detected using a forward 5'-GCGGAGCATATTCAATCACAAG-3' and reverse 5'-TGATGCCAGCTAACATAGAATTGG-3' primer set.

All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Aortic Banding

Left ventricular pressure overload was induced for 28 days by transverse aortic constriction as previously described (39). Briefly, the aorta was ligated between the innominate and left common carotid arteries by tying a 7-0 silk suture around a tapered 27-gauge needle placed on top of the aorta. The needle was removed, and a defined stenosis of the aorta was produced. The skin was closed with separate sutures and buprenorphine was used as an analgesic.

Angiotensin II Infusion

Mice were anesthetized with 1.5% isoflurane and Ang II was infused for 28 days at a rate of 3.2 $mg \cdot kg^{-1} \cdot day^{-1}$ by an osmotic minipump (Alzet) implanted subcutaneously.

Histology

For heart-to-body weight ratios, mice were weighed and hearts were dissected, rinsed in PBS, and weighed. Hearts were then cut transversely just

below the level of the papillary muscle. The top half of the heart was fixed in 4% paraformaldehyde and 5μM sections were prepared. The sections were stained with H&E to assess gross appearance, and Masson's Trichrome staining was used to evaluate fibrosis.

Echocardiography

Transthoracic echocardiography was performed with a 30-MHz probe and the Vevo 660 Ultrasonograph (VisualSonics). Mice were anesthetized with 1-1.5% isoflurane. Heart rate was closely monitored and mice were placed under a heat lamp to maintain a constant body temperature of 37°C. The heart was imaged in a two-dimensional parasternal long-axis view. For measurements, an M-mode cursor was positioned perpendicular to the interventricular septum and the posterior wall of the left ventricle at the level of the papillary muscle.

Cardiomyocyte Contractility Measurements

Adult myocyte isolations were performed as previously described and subsequently used for contractility studies (38). Cells of similar lengths were selected and single-cell contractions were measured in rod-shaped cells by video edge-detection (Crescent Electronics). Percent cell shortening was recorded upon electric field stimulation under basal conditions and following treatment with 1μM isoproterenol. Fifteen cells were analyzed three times for each animal.

Statistics

Statistical analyses were performed with a Student *t* test with unequal variance.

Results

Generation of Cardiomyocyte-Specific *Calcrl* Knockout Mice

To study the cardiomyocyte-specific role of the *Calcrl* gene we generated mice with a "floxed" *Calcrl* allele. As shown in **Figure 3.1A**, we bred *Calcrl*$^{Flox/Flox}$ mice to mice that were heterozygous for the *Calcrl* gene and positive for the *αMHCCre* recombinase transgene, which is specifically expressed in cardiomyocytes (1). By breeding into a *Calcrl*$^{+/-}$ genetic background, we ensured robust excision of a single floxed allele and thereby reduced the incidence of mosaic cellular excision. **Figure 3.1B** shows a typical genotyping PCR with the four genotypes obtained from the above-mentioned cross. For the following experiments, we used *Calcrl*$^{+/LoxP}$*;αMHCCre*$^{+}$ mice as controls and *Calcrl*$^{-/LoxP}$*;αMHCCre*$^{+}$ mice as cardiomyocyte-specific *Calcrl* knockouts. By using mice that are *αMHCCre*$^{+}$ for both controls and knockouts we excluded the possible toxicity of Cre as a basis for our phenotypes. **Figure 3.1C** shows a PCR of genomic DNA samples isolated from hearts and tails. Only DNA isolated from *αMHCCre*$^{+}$ heart samples, in which exons 5 and 6 were excised by Cre-mediated recombination, yielded the 500 bp recombined allele.

As shown in **Figure 3.1D**, all four genotypes generated from our crosses were observed in the expected Mendelian ratios, including the cardiomyocyte-specific *Calcrl* knockouts. All of these mice survived well into adulthood with no

obvious phenotypic defects. Therefore, we conclude that the expression of *Calcrl* in cardiomyocytes is not required for normal embryonic development and survival.

We also generated cardiomyocyte-specific *Calcrl* knockouts by crossing our mice to the cardiac troponin Cre (*cTNTCre*) line and observed similar results (data not shown). Therefore, we conducted the rest of our studies using only the *αMHCCre* line.

Cardiac Function in Cardiomyocyte-Specific *Calcrl* Knockouts

To assess functional and morphological parameters under basal conditions, anesthetized 12-15 week old male mice were subjected to echocardiography. **Table 3.1** shows that none of the physiological parameters we measured were significantly different between *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ mice. All of the measured parameters fell within the range of previously reported values (3, 24, 38).

Response of Cardiomyocyte-Specific *Calcrl* Knockouts to TAC

Although cardiac function in cardiomyocyte-specific *Calcrl* knockouts was normal under basal conditions, we were interested in their response to TAC for two main reasons. First, *AM*$^{+/-}$ mice have previously been reported to suffer from greater cardiac hypertrophy and fibrosis following TAC than wild-type controls (35). Second, genes involved in AM signaling, including *Calcrl*, are significantly elevated following TAC-induced pressure overload (37).

Table 3.2 shows heart/body weight ratios and echocardiography data from sham-operated mice and mice that were subjected to TAC for 4 weeks. Although

TAC treatment led to significantly elevated heart/body weight ratios in both genotypes, there were no significant functional differences between *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ mice in response to TAC. The only statistically significant difference observed by echocardiography was the increase in LVPW, d and LV mass in *Calcrl*$^{-/LoxP}$ mice following TAC. Histological analysis of sham-operated animals shows that overall heart size, structure and interstitial fibrosis (Masson's Trichrome) were similar in *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ mice **(Figure 3.2A-3.2D)**. Furthermore, histology shows that TAC resulted in enlarged left ventricular size and increased interstitial fibrosis, with no significant differences between the two genotypes **(Figure 3.2E-Figure 3.2H)**.

We also monitored the survival rate over the 4 week course of TAC challenge. As shown by the Kaplan-Meier survival curve in **Figure 3.3**, approximately 30% of the animals died following TAC. There were no significant differences in mean survival times or percent survival between *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ mice. All animals that underwent sham surgeries survived (data not shown).

Therefore, from these studies we conclude that loss of expression of *Calcrl* in cardiomyocytes does not substantially impact on the homeostatic response to pressure-induced cardiac hypertrophy.

Response of Cardiomyocyte-Specific *Calcrl* Knockouts to Angiotensin II

Niu *et al*. showed that cardiac hypertrophy induced by Ang II was more severe in *AM*$^{+/-}$ mice than wild-type controls (34, 35). We therefore hypothesized

that cardiomyocyte-specific *Calcrl* knockouts might show a similar, more extreme, response when challenged with Ang II.

Table 3.3 shows heart/body weight ratios and cardiac function in *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ animals after four weeks of Ang II treatment administered by osmotic minipumps. While Ang II caused marked cardiac hypertrophy compared to sham and untreated animals (data not shown), there were no significant differences observed in any of the functional parameters measured. Furthermore, histological analysis revealed no obvious differences in heart size, structure or fibrosis between the two genotypes after four weeks of Ang II challenge **(Figure 3.4)**. No animals died in the Ang II experiments (data not shown).

We thus conclude that loss of *Calcrl* expression in cardiomyocytes does not significantly affect the heart's response to Ang II-induced cardiac hypertrophy.

Assessment of Cardiomyocyte Contractility

To test the role of *Calcrl* in regulating contractility in a genetic model, we used single cell suspensions of cardiomyocytes from *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ animals and measured percent cell shortening under basal and isoproterenol (1μM) stimulated conditions. We found no significant differences between *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ cardiomyocytes in percent cell shortening under basal conditions. As expected, isoproterenol treatment significantly increased percent cell shortening in myocytes of both genotypes, but no significant differences were observed between the two groups **(Figure 3.5)**.

Therefore, we conclude that loss of *Calcrl* expression does not substantially affect basal or isoproterenol-stimulated cardiomyocyte contractility.

Discussion

AM is a multifunctional peptide that is secreted by several cell types including endothelial cells, vascular smooth muscle cells and cardiomyocytes (11). Of particular interest in the AM field is its role in cardiovascular physiology and disease. Multiple studies in both animals and humans have strongly supported a protective role for AM in heart disease (7, 16, 27, 28, 34, 35). However, our understanding of AM physiology is still incomplete due to several factors, including embryonic lethality of *AM* and *Calcrl* knockouts and a broad expression pattern of *AM* and genes involved in its signaling. To better understand the role of AM signaling in cardiac development and physiology, we generated mice with a "floxed" *Calcrl* allele and crossed them to *αMHC* Cre mice, resulting in deletion of the *Calcrl* gene specifically in cardiomyocytes. We chose to delete *Calcrl*, rather than *AM*, because AM is secreted by various cell types and acts in a paracrine manner, so that its genetic excision in one cell type may not preclude its activity within that cell. By generating mice with a conditional deletion of the AM receptor, *Calcrl*, the loss of AM signaling in a specific cell type can be readily addressed.

Since the original *Calcrl*$^{+/-}$ mice have no obvious phenotypic defects (9), we were able to incorporate them into our breeding strategy for generating cardiomyocyte-specific *Calcrl* knockouts. *Calcrl*$^{+/Flox}$ and *Calcrl*$^{-/Flox}$ mice only required one floxed allele to be deleted by Cre-mediated recombination, therefore

limiting the possibility of mosaic cellular excision. Successful Cre-mediated cardiomyocyte-specific deletion of *Calcrl* was confirmed by detection of the null allele exclusively in the myocardium of *αMHCCre*$^{+}$ *Calcrl*-floxed mice. Four genotypes were obtained from our breeding strategy and all of them were observed at the expected Mendelian ratios well into adultdhood. Thus, we conclude that the specific loss of *Calcrl* in cardiomyocytes is not the cause of generalized edema or embryonic lethality previously observed in global *Calcrl* knockout mice. Consistent with this conclusion is the observation that loss of *Calcrl* in endothelial cells leads to an embryonic lethal phenotype similar to that observed in the global knockouts (JCI in press).

The viability of *Calcrl*$^{-/LoxP}$ mice allowed us to address the role of AM signaling in cardiomyocytes of normal and challenged adult animals. We performed echocardiography in cardiomyocyte-specific *Calcrl* knockouts under basal conditions to evaluate cardiac structure and function. Structural and functional characteristics of cardiomyocyte-specific knockouts were strikingly similar to controls, with no significant differences between any of the measured parameters.

Although basal cardiac function was normal in cardiomyocyte-specific *Calcrl* knockouts, we hypothesized that their response to cardiac challenge might be altered. Several lines of evidence led us to propose this theory. First, AM peptide levels are significantly elevated in human heart disease and in various animal models of cardiac challenge (17, 18, 22, 30-32). Second, AM has consistently been shown to be cardioprotective in a number of disease conditions, especially heart disease. AM serves several protective mechanisms, including inhibition of fibrosis

and collagen production by fibroblasts (16, 35), increasing coronary blood flow (27), reducing blood pressure (15, 23, 29), enhancing cardiomyocyte contractility (13) and reducing plasma aldosterone levels (6). Finally, genes involved in AM signaling, including *Calcrl*, are dynamically regulated in the myocardium during cardiovascular stress (8, 32, 45). Therefore, it was somewhat surprising that the cardiomyocyte-specific *Calcrl* knockouts and controls responded similarly to cardiac challenge induced by aortic constriction or Ang II infusion. As our results show, there were no significant differences between knockouts and controls in cardiac structure, function or survival in either of the models tested. From these data we conclude that signaling for AM in cardiomyocytes does not represent the major protective function of this peptide to cardiovascular damage.

The role of AM signaling in regulating cardiomyocyte contractility is somewhat controversial. Although this topic has been investigated in great detail by others (13, 14, 26, 42), this study is the first to use a genetic model. By measuring percent cell shortening in individually isolated myocytes, we concluded that there were no significant differences in contractility between control and knockout cells.

The results of our study are surprising. The AM peptide seemingly plays a major role in cardiovascular biology, but knocking out its primary receptor in cardiomyocytes has no effect? There are a few possible explanations for this observation. One is that AM signaling through *Calcrl* is more important in other cell types, such as endothelial cells or vascular smooth muscle cells. Indeed, we have recently reported that mice lacking *Calcrl* specifically in endothelial cells suffer from vascular defects and generalized edema which ultimately leads to death at mid-

gestation, similar to global *AM* and *Calcrl* knockouts (JCI in press). Therefore, it is possible that the cardioprotective effects observed in humans or animals treated with AM occur in response to a primary effect of AM on the vasculature. Furthermore, most genetic animal models reported to date disrupt AM expression in all tissues. Since the disease models we have used in this study are complex and involve multiple cardiovascular components, this may be a reason we did not observe significant differences in cardiomyocyte-specific knockouts. Another possible explanation which we cannot rule out is the possibility of another AM receptor in cardiomyocytes which may compensate for the loss of *Calcrl*. Other putative AM receptors have been suggested to mediate the biological actions of AM (2, 20, 21).

This study was the first to examine the cardiomyocyte-specific role of the AM receptor, CLR. We show that *Calcrl* expression in cardiomyocytes is not required for embryonic survival or for the cardioprotective functions of AM in response to pressure overload or Ang II-induced cardiovascular disease of adult animals.

Figure 3.1

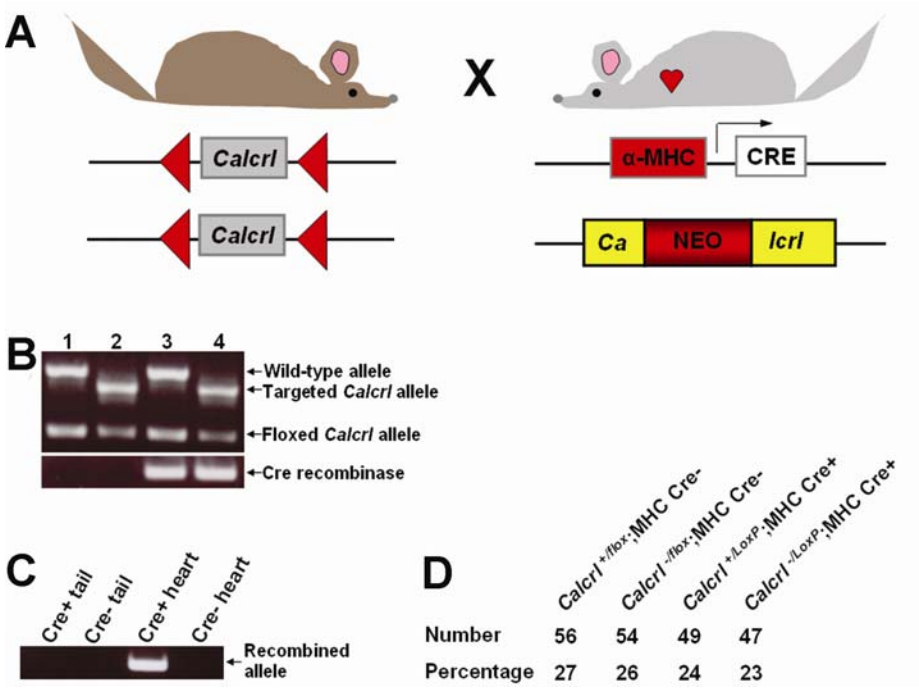

Figure 3.1: Generation of cardiomyocyte-specific *Calcrl* knockout mice. (A) Schematic representation of the breeding scheme used to generate mice with *Calcrl* expression deleted specifically in cardiomyocytes by use of the αMHCCre transgene. (B) Typical genotyping PCR showing the four possible genotypes which can result from the crosses depicted in 1A. (C) PCR from tail or heart genomic DNA shows that the recombined allele which results from Cre-mediated recombination is only detected in Cre+ cardiac tissue. (D) Results from cross demonstrate that all four possible genotypes are observed at the expected Mendelian ratios.

Figure 3.2

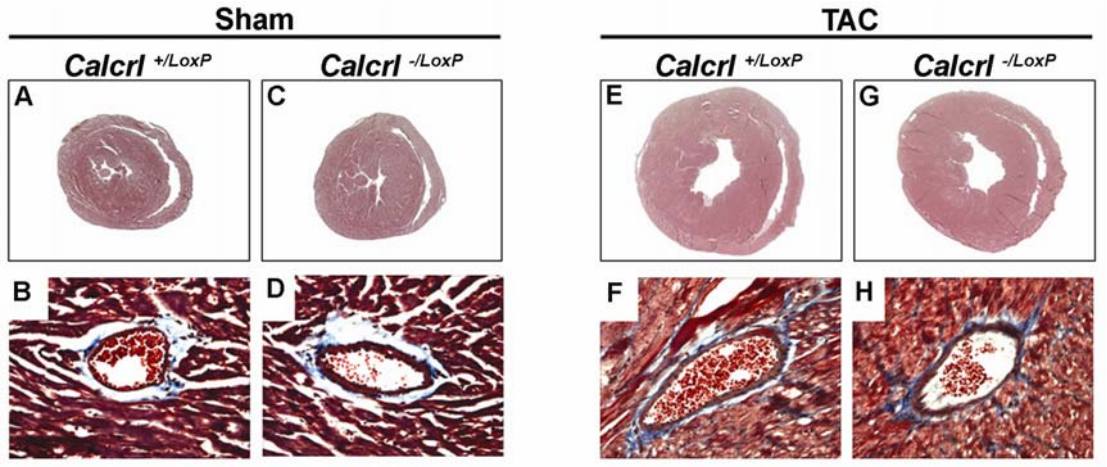

Figure 3.2: Histological examination of the heart. (A&C) H&E stained hearts from sham operated animals. (B&D) Masson's trichrome stained hearts of sham operated animals. (E&G) H&E stained hearts from TAC animals. (F&G) Masson's trichrome stained hearts from TAC animals.

Figure 3.3

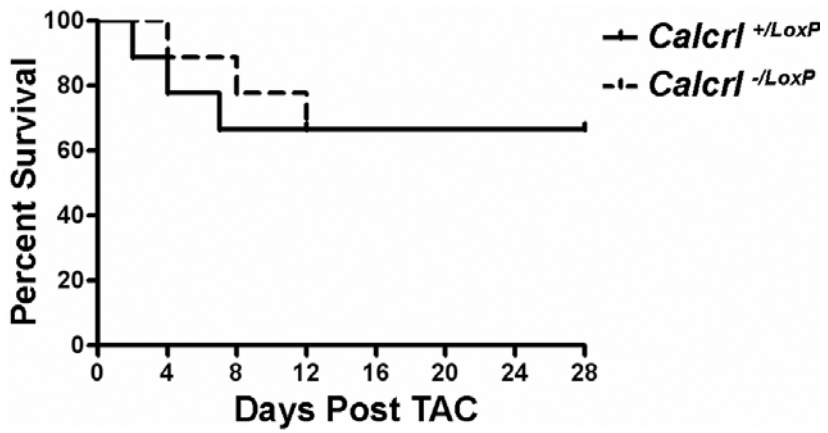

Figure 3.3: Survival rate after aortic constriction. Percent survival after 28 days of aortic constriction. No significant differences were observed between the two groups.

Figure 3.4

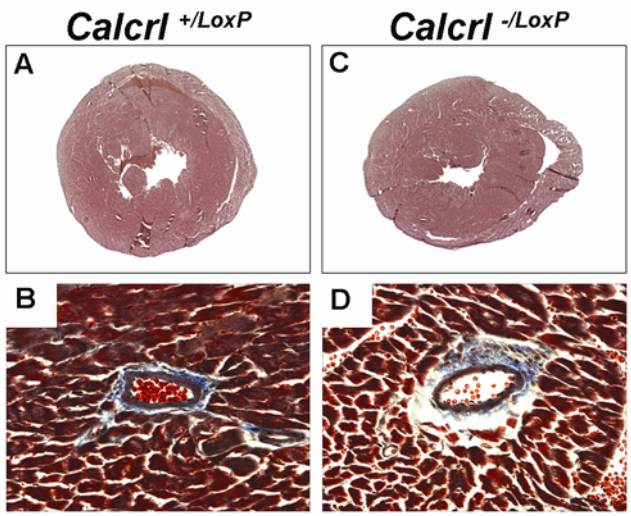

Figure 3.4: Histological examination of Ang II treated hearts. (A&C) H&E stained hearts from Ang II treated animals. (B&D) Masson's trichrome stained hearts from Ang II treated animals.

Figure 3.5

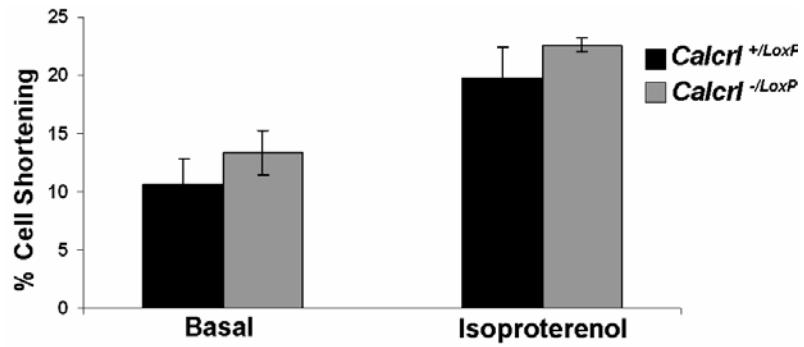

Figure 3.5: Cardiomyocyte contractility. Basal and 1 μM isoproterenol-induced percent cell shortening of *Calcrl*$^{+/LoxP}$ and *Calcrl*$^{-/LoxP}$ cardiomyocytes.

Table 3.1

Table 1
Basal physiological parameters in control and *Calcrl* myocyte-specific knockouts

	Calcrl $^{+/LoxP}$;MHC Cre+	*Calcrl* $^{-/LoxP}$;MHC Cre+	p value
N	11	11	
Heart Rate (bpm)	446 ± 17	446 ± 16	1.00
LVED, d (mm)	4.04 ± 0.13	3.95 ± 0.11	0.56
LVED, s (mm)	3.03 ± 0.16	2.91 ± 0.15	0.55
LVPW, d (mm)	0.74 ± 0.05	0.79 ± 0.07	0.35
LVPW, s (mm)	1.03 ± 0.04	1.11 ± 0.05	0.23
% FS	25 ± 2	26 ± 2	0.69
%EF	49 ± 3	51 ± 4	0.71
CO (ml/min)	16 ± 1	15 ± 1	0.89
LV Mass (mg)	121 ± 9	117 ± 6	0.73

Table 3.2

Table 2
Physiological parameters in control and *Calcrl* myocyte-specific knockouts after four weeks of transverse aortic constriction (TAC)

	Calcrl $^{+/LoxP}$**;MHC Cre+**		***Calcrl*** $^{-/LoxP}$**;MHC Cre+**	
	Sham	**TAC**	**Sham**	**TAC**
N	4	6	4	6
Heart/Body Weight	4.41 ± 0.26	7.89 ± 0.89*	4.33 ± 0.24	7.06 ± 0.80*
Heart Rate (bpm)	494 ± 46	462 ± 23	449 ± 33	463 ± 17
LVED, d (mm)	3.93 ± 0.17	4.04 ± 0.24	3.89 ± 0.19	3.98 ± 0.17
LVED, s (mm)	3.08 ± 0.14	3.28 ± 0.35	2.95 ± 0.29	3.22 ± 0.20
LVPW, d (mm)	0.91 ± 0.17	1.12 ± 0.11	0.82 ± 0.06	1.13 ± 0.08*
LVPW, s (mm)	1.23 ± 0.21	1.38 ± 0.12	1.22 ± 0.10	1.35 ± 0.08
%FS	21 ± 3	19 ± 4	24 ± 4	18 ± 2
%EF	43 ± 6	39 ± 8	47 ± 8	39 ± 4
CO (ml/min)	15 ± 3	12 ± 2	14 ± 2	12 ± 1
LV Mass (mg)	154 ± 17	170 ± 24	119 ± 3	193 ± 22*

*P<0.05 in sham vs. TAC within the same genotype.

Table 3.3

Table 3
Physiological parameters in control and *Calcrl* myocyte-specific knockouts following four weeks of angiotensin II treatment.

	Calcrl $^{+/LoxP}$;MHC Cre+	*Calcrl* $^{-/LoxP}$;MHC Cre+	p value
N	4	4	
Heart/Body Weight	6.71 ± 0.15	6.56 ± 0.69	0.82
Heart Rate (bpm)	451 ± 35	529 ± 37	0.08
LVED, d (mm)	3.39 ± 0.14	3.65 ± 0.43	0.55
LVED, s (mm)	2.48 ± 0.17	2.58 ± 0.45	0.82
LVPW, d (mm)	1.23 ± 0.04	1.20 ± 0.06	0.66
LVPW, s (mm)	1.54 ± 0.11	1.48 ± 0.06	0.58
% FS	27 ± 3	29 ± 4	0.69
%EF	53 ± 4	57 ± 6	0.62
CO (ml/min)	11 ± 1	17 ± 3	0.16
LV Mass (mg)	143 ± 14	148 ± 23	0.86

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CHAPTER 4

receptor activity modifying proteins 2 AND *3* HAVE DISTINCT PHYSIOLOGICAL FUNCTIONS FROM EMBRYOGENESIS TO OLD AGE

Abstract

Receptor activity modifying proteins (RAMPs) impart remarkable effects on G-protein coupled receptor (GPCR) signaling. First identified through an interaction with the calcitonin receptor-like receptor (CLR), these single transmembrane proteins are now known to modulate the in vitro ligand binding affinity, trafficking and second messenger pathways of numerous GPCRs. Consequently, the receptor-RAMP interface represents an attractive pharmacological target for the treatment of disease. Although the three known mammalian RAMPs differ in their sequences and tissue expression, results from in vitro biochemical and pharmacological studies suggest that they have overlapping effects on the GPCRs with which they interact. Therefore, to determine whether RAMP2 and RAMP3 have distinct functions in vivo we generated mice with targeted deletions of either the *Ramp2* or *Ramp3* gene. Strikingly, we find that while *Ramp2* is required for survival, mice that lack *Ramp3* appear normal until old age, at which point they have decreased weight. In addition, mice with reduced expression of *Ramp2*, but not *Ramp3*, display remarkable sub-fertility. Thus, each gene has functions in vivo that cannot be accomplished by the other. Because RAMP2, RAMP3 and CLR transduce the signaling of two potent vasodilators, adrenomedullin and calcitonin gene related peptide, we tested the effects of our genetic modifications on blood pressure and no effects were detected. Nevertheless, our studies reveal that RAMP2 and RAMP3 have distinct physiological functions throughout embryogenesis, adulthood and old age, and the mice we have generated provide novel genetic tools to further explore the utility of the receptor-RAMP interface as a pharmacological target.

Introduction

The identification of receptor activity modifying proteins 1-3 (RAMPs) has revolutionized our current understanding of the mechanism through which class II G-protein coupled receptors (GPCRs) bind to their peptide ligands. First identified in association with the calcitonin receptor-like receptor (CLR, formerly called CRLR), either of these three single-pass transmembrane proteins can bind to a GPCR, chaperone it to the plasma membrane and alter the ligand binding affinity of the receptor (23). For example, a CLR/RAMP1 complex preferentially and specifically binds to calcitonin gene related peptide (CGRP) while a CLR/RAMP2 or CLR/RAMP3 complex will preferentially bind to adrenomedullin (AM), another peptide vasodilator. Thus, the different spatial and temporal expression patterns of RAMP1, RAMP2 and RAMP3 determine how a cell or tissue will sense and respond to either extracellular CGRP or AM.

Biochemical studies using heterologous over-expression of RAMPs in cultured cells have demonstrated that this general mechanism also applies to several other GPCRs of the class II family, including calcitonin (CT) receptor, parathyroid receptors 1 and 2, vasointestinal peptide/pituitary adenylate cyclase activating peptide 1 (VIP/VPAC1) receptor, and glucagon receptor (4, 5). More recently, Bouschet et al. also demonstrated that RAMP1 or RAMP3 can functionally target a class III receptor, the calcium sensing receptor, to the plasma membrane (3). Therefore it is likely that RAMP proteins have evolved to impart a highly controllable mechanism for modulating GPCR signaling that may be broadly applicable to many GPCRs (9). As a consequence, the pharmacological and

biochemical study of the RAMP-receptor interaction has been geared towards identifying compounds that exploit this interface as a potential drug target for the specific modulation of GPCR signaling for the treatment of human disease (15). One such compound, BIBN4096BS which is currently in clinical trials for the treatment of migraine, acts as a selective CGRP antagonist by interfering with the hCLR/hRAMP1 interaction (7, 8). Yet, the developmental and physiological consequences of genetically altering RAMP function or expression in the whole animal have not been addressed experimentally.

Receptor-associated RAMPs have been linked with receptor glycosylation, receptor trafficking, ligand binding and alteration of second messenger signaling (recently reviewed (10, 13)). However, these ascribed cellular functions are not consistently conserved among the different receptor-RAMP complexes and are highly affected by the choice of cell type and species of RAMPs studied (13). Moreover, pharmacological studies with the most well-characterized receptor-RAMP pairs, CLR/RAMP1-3 and CT/RAMP1-3, demonstrate varying degrees of overlap in the absolute ligand binding affinity imparted by the different RAMPs. For example, CLR/RAMP1-3 complexes can all bind AM or CGRP, but with different affinities (12, 18, 27), and CT/RAMP1-3 complexes can form amylin receptors with highly variable affinities (5, 26) or CGRP receptors (11, 19) depending on the cell type studied. Thus, it remains unclear to what degree the three mammalian RAMPs have overlapping functions or whether they can functionally compensate for each other in vivo.

To address these questions, we used gene targeting to generate mice with targeted deletions of either the *Ramp2* or *Ramp3* genes, and have determined the effects of their complete absence (in homozygous null mice) and of their reduced expression (in heterozygous mice).

Experimental Procedures

Generation of mice with targeted deletion of the *Ramp2* or *Ramp3* gene

To generate the targeting vectors, a 129S6/SvEv genomic library was screened for phage clones containing the 5' portions of the *Ramp2* and *Ramp3* genes using DNA fragments isolated from h*Ramp2* or h*Ramp3* expression plasmids (kindly provided by Dr. Steven Foord, GlaxoSmithKline, UK). Using convenient restriction sites within the genomic clones, 5' and 3' regions of homology were subcloned into the multiple cloning site of a gene targeting vector (osdupdel) which contained a PGK-neomycin cassette and an a HSV-thymidine kinase cassette. The final targeting vectors were linearized with Not I before electroporation into embryonic stem (ES) cells.

Standard gene targeting methods were utilized to generate ES cells and mice with a targeted deletion of the *Ramp2* gene or a targeted deletion of the *Ramp3* gene (16). Briefly, 129S6/SvEv-TC-1 ES cells were electroporated with the linearized targeting vectors shown in Fig. 4.1a and 4.2a, respectively. After applying positive (G418) and negative (ganciclovir) selection, positive ES cell clones were identified by Southern blot and/or PCR. The frequency of homologous recombination in the surviving G418/gancyclovir resistant colonies was 5% for

Ramp2 and 1.5% for *Ramp3*. Male chimeric mice that transmitted the targeted allele were bred to 129S6/SvEv females to establish isogenic lines.

For PCR-based genotyping of the *Ramp2* targeted locus we used three primers: primer 1: 5'- CTGAACTGAACAGCAGGGCCA-3'; primer 2: 5' CGGCTACTTCCCACTTAATGCTG-3'; primer 3: 5'GCTTCCTCTTGCAAAACCACA-3'. Primers 1 and 3 amplify a 1.2 kb targeted band while primers 1 and 2 amplify a 1.6 kb wildtype band.

For PCR-based genotyping of the *Ramp3* targeted locus we used four primers: primer 1: 5'-GCCCATGATGTTGGTCTCCA -3'; primer 2: 5' GGTCATTAGGAGCCACGTGT-3'; primer 3: 5'GCTTCCTCTTGCAAAACCACA-3'; primer 4: 5'GGGCTAAAGAAGCCACAGCT-3'. Primers 1 and 3 amplify a 2.0 kb targeted band while primers 2 and 4 amplify a 1.4 kb wildtype band.

Gene Expression Analysis

Ramp2, *Ramp3* and *Calcrl* gene expression was analyzed by quantitative RT-PCR with the Mx3000P Q-PCR machine from Stratagene. Primers for *Ramp2* amplification were 5'-CAGAATCAATCTCATCCCACTGAC-3' and 5'-GTCCATGCAACTCTTGTACTCATACC-3'. The probe sequence for *Ramp2* detection was 5'-FAM-ATGGAAGACTACGAAACACATGTCCTACCTTG-TAMRA-3'. Primers for *Ramp3* amplification were 5'-GGTCATTAGGAGCCACGTGT-3' and 5'-GGGCTAAACAAGCCACAGCT-3'. The probe sequence for *Ramp3* detection was 5'-FAM-CACGATTCTGTGTCCAGTGTGGGCTG-TAMRA-3'. Primer and probe sequences for detection of *Calcrl* gene expression were previously described (6). -

actin served as an internal control for all reactions. The primers used for □-actin amplification were 5'-CTGCCTGACGGCCAAGTC-3' and 5'-CAAGAAGGAAGGCTGGAAAAGA-3'. The probe sequence for β-actin detection was 5'-TET-CACTATTGGCAACGAGCGGTTCCG-TAMRA-3'. RNA was isolated from adult tissues or E13.5 embryos with TRIzol Reagent (GIBCO/BRL) and subsequently DNase treated and purified with an RNeasy Mini Kit (Qiagen). 200 ng of total RNA was used in each reaction. The ΔΔCt method (21) was used to determine the relative levels of gene expression and shown as a percentage of wild type. All assays were repeated three times, each with duplicates.

Measurement of Basal Blood Pressure, Heart Rate and Urine Chemistry

Blood pressures and heart rates were measured on unanesthetized mice by a computerized tail cuff system (17). Urine and protein creatinine were measured at UNC-CH's Animal Clinical Chemistry Core Facility.

Experimental Animals

Unless otherwise noted, experimental animals were 4-8 months old and maintained on an isogenic 129S6/SvEv-TC-1 background. Control animals for all experiments consisted of wildtype, age- and gender-matched littermates. All experiments were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Statistics

Statistical analyses for multiple comparisons were performed with Oneway ANOVA by JMP Software, SAS Institute. Error bars represent standard error of the means. Differences were considered significant with a p value of < 0.05.

Results

Generation of heterozygous mice with targeted disruption of the *Ramp2* gene

Mice in which exons 1 and 2 of the *Ramp2* gene were deleted by homologous recombination were generated using the targeting strategy shown in Figure 4.1A. The disrupted allele, which lacks the *Ramp2* promoter, 5' untranslated region, translation start site and exons 1 and 2, was confirmed by genomic PCR (Fig. 4.1B). To confirm that the gene targeting effectively disrupted transcription of the *Ramp2* gene, quantitative reverse transcription-PCR for *Ramp2* RNA was performed on total RNA isolated from whole embryos. As expected and shown in Figure 4.1C, *Ramp2*$^{+/-}$ embryos expressed approximately half of wildtype *Ramp2* RNA levels (58%, $p< 0.001$ vs. wildtype) while *Ramp2*$^{-/-}$ embryos had no detectable levels of *Ramp2* RNA, thus confirming correct gene targeting of the *Ramp2* gene (Figure 4.1C). To determine whether genetic reduction of *Ramp2* caused a homeostatic compensation in the expression of the *Ramp3* or *Calcrl* genes, we measured the expression of these genes in the heart and kidneys of adult *Ramp2*$^{+/-}$ animals and in total RNA isolated from mid-gestation *Ramp2*$^{-/-}$ embryos. As shown in Figure 4.1D, we found no significant compensatory increase in the expression of either *Ramp3* or *Calcrl* in *Ramp2*$^{+/-}$ or *Ramp2*$^{-/-}$ mice compared to wildtype controls, demonstrating that the

genetic reduction of *Ramp2* does not result in a compensatory up-regulation of *Ramp3* or *Calcrl* gene expression.

Generation of mice lacking the *Ramp3* gene

Homozygous null mice for the *Ramp3* gene were generated by homologous recombination using the targeting strategy shown in Figure 4.2A. The disrupted allele, which lacks exons 2 and 3 of the *Ramp3* gene (coding for amino acids 19-147 of 147 total amino acids), was detected by Southern blot analysis using a genomic probe fragment located outside the areas of homology (Fig. 4.2B) and by genomic PCR (Fig. 4.2C). The correctly targeted allele was further confirmed by direct sequencing (data not shown). To confirm that the gene targeting effectively disrupted transcription of full length *Ramp3* mRNA, quantitative reverse transcription-PCR for *Ramp3* mRNA was performed on total RNA isolated from adult kidneys. As expected, *Ramp3*$^{+/-}$ mice expressed approximately half of wildtype *Ramp3* RNA levels (45%, $p < 0.0001$ vs. wildtype) while *Ramp3*$^{-/-}$ mice had no detectable levels of *Ramp3* RNA, thus confirming complete loss of *Ramp3* expression in adult homozygous mice (Fig. 4.2D). To determine whether genetic deletion of *Ramp3* caused a homeostatic compensation in the expression of the *Ramp2* or *Calcrl* genes in adult animals, we measured the expression of these genes in the heart and kidneys of *Ramp3*$^{-/-}$ animals. As shown in Figure 4.2E, we found no significant compensatory increase in the expression of either *Ramp2* or *Calcrl* genes in *Ramp3*$^{-/-}$ mice compared to wildtype controls, demonstrating that the

genetic deletion of *Ramp3* does not impact on the regulation of *Ramp2* or of *Calcrl* gene expression.

***Ramp2* is essential for survival and normal fertility**

In marked contrast, while the loss of *Ramp3* did not affect the survival of *Ramp3*$^{-/-}$ mice to adulthood, we found that genetic loss of *Ramp2* caused embryonic lethality since no *Ramp2*$^{-/-}$ pups were born alive to heterozygote matings. This remarkable contrast in phenotypes (embryonic lethality of *Ramp2*$^{-/-}$ mice versus survival of *Ramp3*$^{-/-}$ mice) demonstrates that these two genes have significantly different functions during embryonic development.

Offspring from *Ramp3*$^{+/-}$ matings were born in the expected Mendelian ratio of 1:2:1 (26 *Ramp3*$^{+/+}$:48 *Ramp3*$^{+/-}$:25 *Ramp3*$^{-/-}$ pups from 15 litters) and had litter sizes comparable to isogenic control matings (6.6 pups/litter for *Ramp3*$^{+/-}$ matings versus 6.0 pups/litter for 129S6/SvEv isogenic control matings, Figure 4.3). In contrast, although the ratio of wildtype:heterozygote genotypes for viable pups born to *Ramp2*$^{+/-}$ matings was in the expected Mendelian distribution of 1:2 (22 *Ramp2*$^{+/+}$:44 *Ramp2*$^{+/-}$ pups from 29 litters), the average litter size was significantly reduced to 2.1 pups per litter at wean. This markedly reduced litter size is significantly below the expected litter size of 4.5 pups/litter, which takes into consideration the gestational loss of *Ramp2*$^{-/-}$ null embryos (further confirmed by the assessment of litter sizes for *Calcrl* heterozygote matings in which there is gestational loss of null embryos (6), Figure 4.3). Thus, while genetic loss of *Ramp3* was dispensable for normal fertility, a modest genetic reduction of *Ramp2* was sufficient to cause marked sub-fertility,

demonstrating that the two genes maintain distinct physiological functions during adulthood.

Aged *Ramp3*$^{-/-}$ mice fail to gain weight

Ramp3$^{-/-}$ mice survived to adulthood, reproduced and displayed no obvious phenotypic defects until ~6 months of age. Although the body weights of young *Ramp3*$^{-/-}$ mice did not differ from their wildtype controls up to ~6 months of age (Table 4.1), we noticed that older *Ramp3*$^{-/-}$ mice (9-10 months of age) weighed nearly 9 grams less than age-matched wildtype mice (Figure 4.4B, wildtype mice weighed 36.1 +/- 1.9 g versus 27.3 +/- 1.1 g for age-matched *Ramp3*$^{-/-}$ mice). In contrast, aged *Ramp2*$^{+/-}$ mice did not differ significantly in body weights from their wildtype littermates (Figure 4.4A, wildtype weighed 28.3 +/- 0.5 g versus 28.8 +/- 0.7 g for age-matched *Ramp2*$^{+/-}$ mice). Despite their visibly lean appearance, we found no significant differences in food or water intake in either young or aged *Ramp3*$^{-/-}$ mice compared to their age-matched, wildtype controls (Table 4.1). Moreover, *Ramp3*$^{-/-}$ mice, like their wildtype counterparts, survived to at least 18 months of age with no obvious decline in health. Since *Ramp3* is highly expressed in the proximal tubule of the kidney, we also compared urine volume and kidney function (as determined by protein/creatinine ratio) between *Ramp3*$^{-/-}$ mice and wildtype controls and found no obvious differences (Table 4.1). The body weights, feeding behavior and kidney function of *Ramp2*$^{+/-}$ mice or *Calcrl*$^{+/-}$ mice did not differ from wildtype control littermates (Table 4.1).

Blood pressures and heart rates are unaffected in *Ramp2*$^{+/-}$ and *Ramp3*$^{-/-}$ mice

Because CLR is the best characterized receptor partner for RAMP2 and RAMP3 and because CLR binds to two potent peptide vasodilators (AM and CGRP), we sought to compare the blood pressures and heart rates of *Ramp2*$^{+/-}$ and *Ramp3*$^{-/-}$ mice to those of *Calcrl*$^{+/-}$ (6) and wildtype mice using a computerized tail cuff system. As shown in Table 4.1, we found that reduction of *Ramp2* to ~50% of wildtype levels and complete absence of *Ramp3* had no effect on the basal blood pressures of conscious animals compared to wildtype mice or to *Calcrl*$^{+/-}$ mice. Moreover, we found no overt differences in the heart rates among the genotypes tested (Table 4.1).

Discussion

In summary, we used gene targeting to generate two independent mouse lines with deletion of either the *Ramp2* or *Ramp3* genes. Gene expression analysis in mice with reduced or absent *Ramp2* levels or complete lack of *Ramp3* did not reveal any compensatory up-regulation of either *Ramp3* or *Ramp2* gene expression, respectively, supporting our conclusion that there is no functional redundancy at the transcriptional level between *Ramp2* and *Ramp3* in vivo. We did observe a general trend for significantly reduced expression of *Ramp2*, *Ramp3* and *Calcrl* compared to wildtype mice in the models we tested (Fig. 4.1D and Fig. 4.2E) which is likely reflective of the high sensitivity of these genes to altered physiological homeostasis (18, 27).

Although the biochemical and pharmacological profiles of RAMP2 and RAMP3 appear to overlap for certain GPCRs (CLR, CT and VIP/VPAC1), our genetic studies demonstrate that the two genes have distinct roles throughout the life of an animal. During embryonic development, *Ramp2*$^{-/-}$ mice fail to survive while *Ramp3*$^{-/-}$ mice appear normal up to 6 months of age. During adulthood, loss of *Ramp3* had no apparent effect on fertility. In contrast, a modest genetic reduction of *Ramp2* in heterozygous mice was sufficient to cause a marked reduction in litter size, which is similar to the phenotype we have previously characterized for mice with a genetic reduction of AM (20). Finally, in aged animals we found that *Ramp3*, but not *Ramp2*, plays an important role in maintaining normal body weight, however the physiological mechanisms which account for this phenotype have not yet been resolved.

Our studies to address the regulation of blood pressure and heart rate in these mice are consistent with our recent findings demonstrating that genetic alteration of AM peptide levels from 50%-140% wildtype levels does not affect basal blood pressure. These results are also consistent with another recent study where transgenic over-expression of m*Ramp2* in smooth muscle cells had no effect on basal or induced changes in blood pressure (25). Lu et al have also shown that genetic deletion of α-CGRP does not alter basal blood pressure in mice (22). Taken together, our results indicate that in vivo genetic alteration of *Ramp2*, *Ramp3* or *Calcrl* expression (the receptor signaling components required for transducing the signal of two potent vasodilators, AM and CGRP) does not impact on basal blood pressure regulation in mice. Thus, the use of CLR/RAMP2 or CLR/RAMP3 as

pharmacological targets for the treatment of hypertension in humans should be carefully evaluated.

Our most significant data stem from the direct comparison of phenotypes for the *Ramp2* and *Ramp3* gene targeted mice. While modest changes in the genetic dosage of the *Ramp2* gene have profound effects on survival and reproduction, complete absence of the *Ramp3* gene seems to have little or no effect on mice until old age. These in vivo findings are consistent with the concept that RAMP2 acts to mediate the basal effects of normal GPCR signaling while RAMP3 may become induced under physiological conditions (14) or disease (24) to alter the signaling of GPCRs (10). This concept is further supported by biochemical studies which demonstrate that RAMP3, but not RAMP2, contains an intracellular PDZ motif capable of binding to NSF and NHERF to change receptor internalization and trafficking (1, 2). Thus, the continued study of these genetically engineered mouse models under normal conditions and disease states, as well as cell lines derived from them, will provide useful tools for unraveling the functional role of RAMP2 and RAMP3 in modulating GPCR signaling and testing their potential utility as pharmacological targets for the treatment of human disease.

Figure 4.1

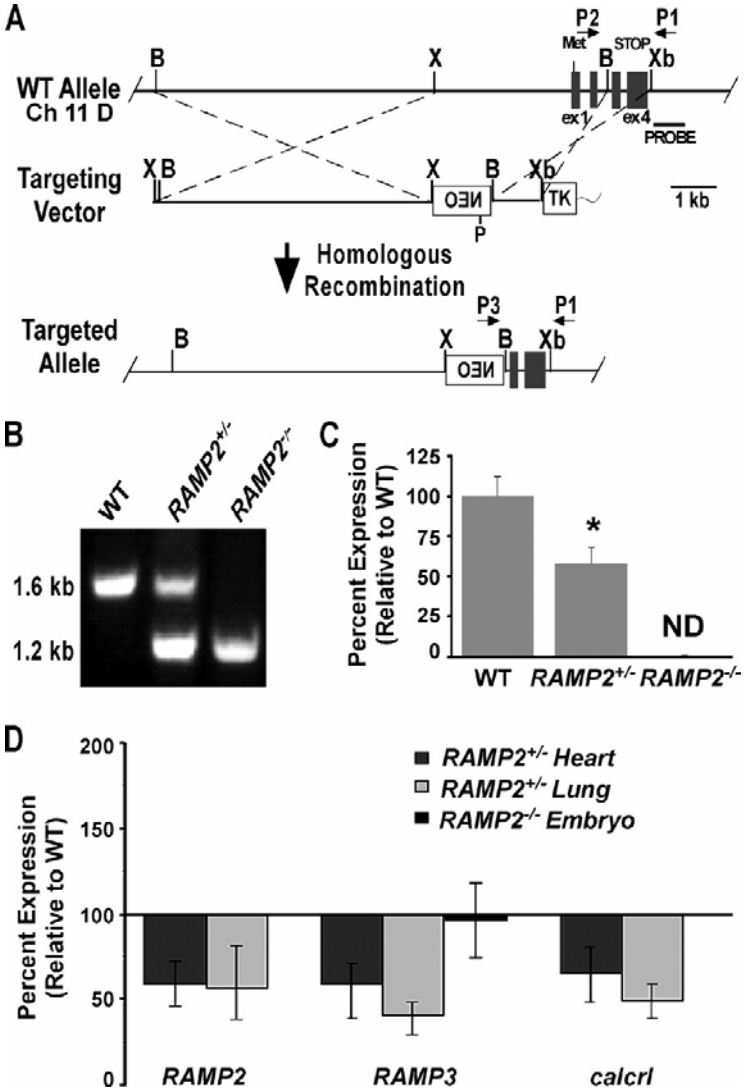

Fig. 4.1. Gene targeting of *Ramp2*. *A*, schematic representation of the strategy used to disrupt the *Ramp2* gene in mice. The promoter region and exons 1 and 2 of *Ramp2*, including the initiator methionine were deleted by homologous recombination. *B*, BamHI X, Xho, Xb, and Xba and P1, P2, and P3 primers used for screening and genotyping. *B*, genomic PCR for detection of the wild type and targeted alleles using total embryonic DNA extracts and the primers depicted in Fig. 4.1*A*. *C*, quantitative RT-PCR for detection of *Ramp2* mRNA transcripts using total embryonic RNA extracts and the primers and probes described under "Experimental Procedures." *ND*, not detected. *D*, quantitative RT-PCR for detection of *Ramp2, Ramp3*, and *Calcrl* mRNA transcripts using total RNA extracted from the hearts and lungs of *Ramp2*$^{+/-}$ adult mice and *Ramp2*$^{-/-}$ embryos.

Figure 4.2

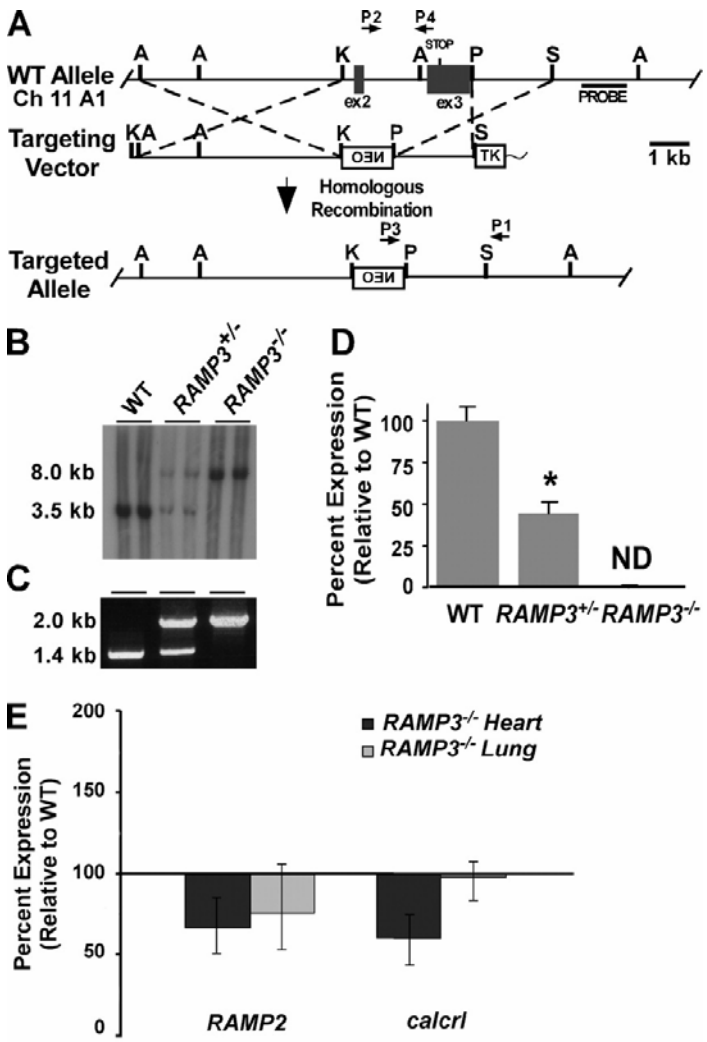

Fig. 4.2. Gene targeting of *Ramp3*. *A*, schematic representation of the strategy used to disrupt the *Ramp3* gene in mice. Exons 2 and 3 of *Ramp3*, including the last 129 amino acids (of 147 total), the stop codon, and the 3'-untranslated region were deleted by homologous recombination. Shown are AvrII (*A*), Kpn (*K*), PvuII (*P*), and SacI (*S*) and P1, P2, P3, and P4 primers used for screening and genotyping. *B*, Southern blot for detection of the wild type and targeted alleles using genomic DNA and the probe fragment depicted in Fig. 4.2*A. C*, genomic PCR for detection of the wild type and targeted alleles using total DNA extracts and the primers depicted in Fig. 4.2*A. D*, quantitative RT-PCR for detection of *Ramp3* mRNA transcripts using total RNA extracted from adult kidneys and the primers and probes described under "Experimental Procedures." *ND*, not detected. *E*, quantitative RT-PCR for detection of *Ramp2* and *Calcrl* mRNA transcripts using total RNA extracted from the hearts and lungs of *Ramp3*$^{-/-}$ adult mice.

Figure 4.3

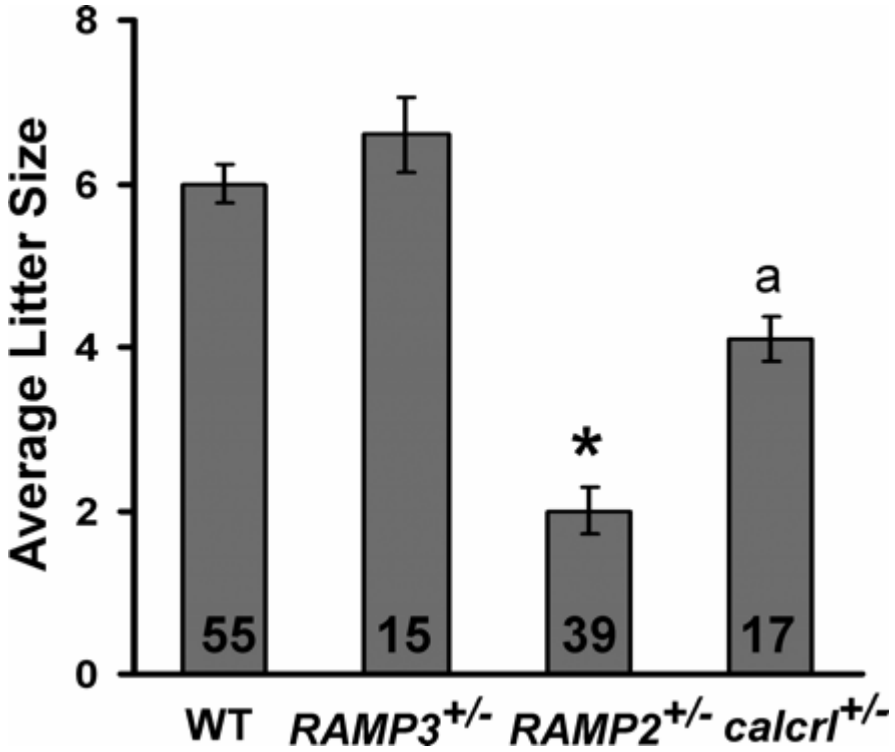

Fig. 4.3. Severely reduced fertility in *Ramp2*$^{+/-}$ mice. Average litter sizes at weaning resulting from heterozygote matings of the genotype indicated. The *number* at the *bottom* of each bar represents the total number of litters. Litter size of *Calcrl*$^{+/-}$ matings is significantly reduced because of the previously reported embryonic lethality of *Calcrl*$^{-/-}$ embryos (18). *, $p < 0.05$ by analysis of variance.

Figure 4.4

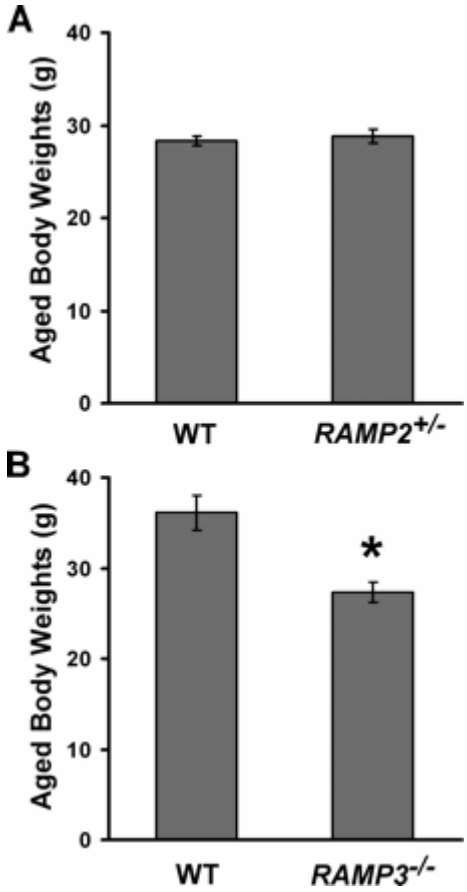

Fig. 4.4. Reduced body weights in aged *Ramp3*$^{-/-}$ mice (but not aged *Ramp2*$^{+/-}$ mice). *A*, body weights of 9–10-month-old *Ramp2*$^{+/-}$ mice compared with their age-matched isogenic controls (Student's *t* test, $p = 0.6$, $n = 7$ for each group). *B*, body weights of 9–10-month-old *Ramp3*$^{-/-}$ mice compared with their age-matched, isogenic controls (Student's *t* test, $p < 0.001$, $n = 10$ for each group).

Table 4.1

Phenotypic analysis of *Ramp2*$^{+/-}$ *and Ramp3*$^{-/-}$ *mice*

	Wild type	*RAMP2*$^{+/-}$	*RAMP3*$^{-/-}$	*calcrl*$^{+/-}$
Body weight (4-6 mo., g)	25.8 ± 0.6	27.3 ± 0.8	24.3 ± 0.5	25.6 ± 0.9
Food intake (24 hr, g)	4.6 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	4.5 ± 0.3
Water intake (24 hr, ml)	3.6 ± 0.2	3.7 ± 0.2	3.4 ± 0.1	3.4 ± 0.2
Urine volume (24 hr, ml)	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
Urine protein/creatinine	0.12 ± 0.02	0.12 ± 0.01	0.08 ± 0.01	0.10 ± 0.03
Mean blood pressure (mm Hg)	106 ± 5	105 ± 3	106 ± 5	111 ± 5
Mean heart rate (beats/min)	641 ± 18	635 ± 9	605 ± 10	591 ± 17
Left ventricle/body weight ratio (3-6 months)	4.11 ± 0.22	4.13 ± 0.14	3.98 ± 0.14	3.97 ± 0.17

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CHAPTER 5

MICE HETEROZYGOUS FOR ADRENOMEDULLIN EXHIBIT A MORE EXTREME INFLAMMATORY RESPONSE TO ENDOTOXIN-INDUCED SEPTIC SHOCK

Abstract

Adrenomedullin (AM) is a highly conserved peptide that can act as a potent vasodilator, anti-microbial factor and anti-inflammatory factor. Several studies have implicated diverse roles for AM in regulating the inflammatory and hemodynamic responses to septic shock. Moreover, during sepsis the receptors that mediate AM signaling [*calcitonin receptor-like receptor* (*Calcrl*) and *receptor activity modifying proteins* (*Ramp*) *2* and *3*] undergo dynamic and robust changes in their expression. Although numerous studies have used animal models to study the role of administered or increased AM in septic animals, genetic studies to determine the consequences of reduced AM during septic shock have not yet been performed. Here, we used a murine model of lipopolysaccharide (LPS)-induced septic shock to assess the inflammatory response in mice heterozygous for the *AM* gene. Following LPS challenge, $AM^{+/-}$ mice had higher expression of *TNF-α* and *IL-1β* than LPS-treated wild-type (WT) controls. Consequently, serum TNF-α was also significantly elevated in LPS-treated $AM^{+/-}$ mice compared to WT LPS-treated mice. We also observed higher serum levels of liver enzymes, suggesting more advanced end-organ damage in mice with genetically reduced AM. Finally, we found that *Ramp2* and *Calcrl* expression levels were markedly reduced in LPS-treated mice, whereas *Ramp3* expression was significantly elevated. Importantly, these changes in receptor gene expression were conserved in $AM^{+/-}$ mice, demonstrating that AM peptide itself does not impact directly on the expression of the genes encoding its receptors. We therefore conclude that during septic shock the dynamic modulation of AM and its receptors primarily functions to dampen the inflammatory response.

Introduction

Adrenomedullin (AM) is a highly conserved gene that may have evolved from an antimicrobial peptide in early eukaryotic organisms into a potent vasodilator in higher mammalian species (34). AM causes relaxation of vascular smooth muscle cells (VSMCs) (6), reduces endothelial cell permeability (9) and is a biologically-relevant antimicrobial peptide involved in the innate immune response (1). The 52-amino acid peptide is produced and secreted by many mammalian tissues and is most highly expressed by VSMCs (27) and endothelial cells (26). Stimuli for AM synthesis and secretion include angiotensin II, endothelin-1, hypoxia, oxidative stress and inflammatory cytokines such as TNF-α and IL-1β (5). Thus, the biological functions of AM in mammals are numerous, diverse and likely inter-related.

Plasma levels of AM are significantly elevated in humans with a wide variety of physiological conditions, including cardiovascular disease, normal pregnancy and septic shock (7). In patients with septic shock, AM peptide levels are 25 to 30 fold higher than in normal individuals (10, 20). Since AM is a potent vasodilator (14), it is reasonable to assume that increased plasma AM contributes to the extreme hypotension observed in the early stages of septic shock. However, our recent studies using genetically engineered mice that lack one copy of the *AM* gene demonstrate that reduction of endogenous AM to 50% of wild-type (WT) levels has no effect on the acute hypotension that occurs in an LPS-induced murine model of septic shock (2). These results suggest that AM may play other primary roles during septic shock.

AM possesses anti-inflammatory (8), bactericidal (1), and positive inotropic (11) properties, which are all beneficial responses to sepsis. When treated with endotoxin, mice over-expressing AM in their vasculature experience less severe hemodynamic and inflammatory responses, less liver damage and lower mortality rates compared to WT endotoxin-treated controls (25). AM has also been shown to reduce TNF-α expression and release in macrophage cell lines and rat Kupffer cells (30). More recently, administration of AM to rats with α-toxin-induced sepsis reduced vascular hyperpermeability and resulted in dramatically improved survival rates (28). Taken together, these results suggest that the beneficial roles of AM during septic shock may primarily be to minimize organ damage by influencing the immune response and/or vascular permeability, rather than by regulating blood pressure. Yet, experiments to genetically confirm the primary function of AM during septic shock have not yet been performed.

The AM peptide contains a 6-residue ring structure and amidated C-terminus which, due to conserved sequence homology and structural motifs, places it in the calcitonin family of peptides, including calcitonin, calcitonin gene related peptide (CGRP), amylin and intermedin (34). Peptides of this family also share a unique mechanism of G-protein coupled receptor signaling by a novel class of single transmembrane proteins called receptor activity modifying proteins (RAMPs). RAMPs were first identified through their association with the calcitonin receptor-like receptor (CLR) and can interact with many other class II GPCRs to determine receptor ligand binding specificity (24). In the case of CLR, association with RAMP1 produces a CGRP receptor, while association with RAMP2 or RAMP3 produces a

receptor specific for AM. In this way, the spatial and temporal expression of RAMP proteins determines the tissue responsiveness to either CGRP or AM.

During inflammation and septic shock, there are robust and dynamic changes in the expression of the *Ramp* and *Calcrl* genes that are responsible for mediating AM signaling. For example, TNF-α significantly reduced the expression of *Calcrl* (the gene encoding CLR), *Ramp1* and *Ramp2* in cultured human coronary artery smooth muscle cells in a time and dose-dependent manner (19). Moreover, Ono et al. have also shown that *Calcrl* and *Ramp2* expression was significantly decreased in lungs of LPS-induced septic mice, while *Ramp3* expression levels were elevated nearly 40-fold (21). In a related fashion, the amount of AM binding protein (AMBP) is significantly reduced during the hypodynamic phase of sepsis, which may account for the reduced responsiveness to elevated plasma AM during the late phase of sepsis (29, 31, 33, 34). These results suggest that the modulation of AM signaling during septic shock is complex (involving both receptor modulation and active peptide bioavailability) and finely tuned in order to maintain homeostatic balance in response to severe physiological insults. However, whether AM signaling itself is involved in these dynamic receptor responses remains unclear.

Our previous studies with genetically engineered mouse models have shown that mice lacking both copies of the *AM* gene or the *Calcrl* gene die at mid-gestation from extreme hydrops fetalis and cardiovascular defects (3, 4). Adult female mice heterozygous for *AM* display profound reproductive defects (15) and are protected from hypertension-induced cardiovascular end organ damage (2). Otherwise, adult male and female *AM* heterozygous mice are born at the expected Mendelian ratios,

survive to adulthood and have normal blood pressures under basal and stressed conditions with no obvious phenotypic defects.

To determine if genetic reduction of endogenous AM affects the septic response in mice, we challenged *AM*$^{+/-}$ mice in an LPS-induced model of septic shock. Since AM is consistently reported as an anti-inflammatory peptide, we were particularly interested in determining whether genetic reduction of endogenous AM in vivo could alter the inflammatory response in septic animals. We also used our genetic model to determine if the dynamic gene expression changes observed in the AM receptor signaling genes during septic shock are dependent on the expression levels of AM peptide.

Experimental Methods

Experimental Animals and LPS Treatment

The generation and phenotype of mice with a targeted deletion of the *AM* gene have been previously described (3). Experiments were carried out with 8-10 week old male mice bred under a controlled environment and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill. Animals used in these experiments were produced by *AM*$^{+/-}$ intercrosses and were maintained on an isogenic 129S6/SvEv genetic background. To induce septic shock in WT and *AM*$^{+/-}$ mice, 60 mg/kg LPS (*Escherichia coli* O55:B5; Sigma, St. Louis, MO) was injected I.P. (unless otherwise stated).

Gene expression analysis

Gene expression was analyzed by quantitative reverse transcription-PCR with the Mx3000P Real-Time PCR machine from Stratagene. Taqman primer and probe sequences for *Calcrl* detection have recently been published. Primers for *Ramp2* were 5'-CAGAATCAATCTCATCCCACTGA-3' and 5'-GTCCATGCAACTCTTGTACTCATAC-3'. The probe for *Ramp2* detection was 5'-FAM-TGGAAGACTACGAAACACATGTCCTACCTTG-TAMRA-3'. Primers for *Ramp3* were 5'-GGTCATTAGGAGCCACGTGT-3' and 5'-GGGCTAAACAAGCCACAGCT-3'. The probe for *Ramp3* detection was FAM-5'-CAGCCCACACTGGACACAGAATCGTG-TAMRA-3'. For *L1* detection, we used a pre-designed, Assays on Demand primer/probe set (Applied Biosystems). Primers for *TNF-α* were 5'-CTGTCTACTGAACTTCGGGGTGAT-3' and 5'-GGTCTGGGCCATAGAACTGATG-3'. The probe for *TNF-α* detection was 5'-FAM-ATGAGAAGTTCCCAAATGGCCTCCCTC-TAMRA-3'. Primer and probe sequences for *IL-1β* detection have been previously published (13). A GAPDH primer/probe set was purchased from Applied Biosystems (part # 4308313) and used as an internal control for all samples. RNA was isolated from lungs with Trizol reagent (Invitrogen), DNase treated, and purified with an RNeasy Mini Kit (Qiagen). 200 ng of RNA was used per reaction and each sample was run in triplicate. The ΔΔCt method was used to determine the relative levels of gene expression (16).

Measurement of serum TNF-α levels

For basal in vivo serum TNF-α concentrations, tail-vein bleeds were performed prior to LPS challenge. Mice were then administered LPS for one hour

and serum samples were again obtained by tail-vein bleed. TNF-α concentrations were quantified using anti-TNF-α antibody by ELISA technique (product # EMTNFA2; Pierce, Rockford, IL). Recombinant TNF-α was used as a standard control.

ALT and AST measurement

Blood chemical analysis for serum alanine (ALT) and aspartate (AST) aminotransferase activities was performed in the Animal Clinical Laboratory Core Facility of the University of North Carolina at Chapel Hill using a Chemical Analyzer VT250 (Ortho-Clinical Diagnostics Comp. Rochester, NY).

Statistical analysis

Statistical analysis was performed using a Student's *t* test with unequal variance. A p value equal to or less than 0.05 was considered statistically significant.

Results

Elevated TNF-α and IL-1β expression in AM$^{+/-}$ septic mice

Using quantitative RT-PCR, the expression of the pro-inflammatory cytokines *TNF-α*, *IL-1β*, and *IL-6* was measured in WT and *AM*$^{+/-}$ mice treated with or without LPS for 45 minutes. Both WT and *AM*$^{+/-}$ animals showed a robust and rapid increase in *TNF-α* expression following LPS administration. However, induction of *TNF-α* expression was significantly higher in lungs of *AM*$^{+/-}$ mice than in lungs of WT control mice (Fig. 5.1). Similarly, *IL-1β* expression was drastically elevated in both

WT and *AM*$^{+/-}$ mice upon LPS challenge, and this increase was significantly higher in *AM*$^{+/-}$ mice than in WT mice (Fig. 5.1). Although the expression of *IL-6* was higher in *AM*$^{+/-}$ mice than in WT controls, this data did not reach statistical significance (data not shown). These results demonstrate that the lungs of mice with only one copy of the *AM* gene (*AM*$^{+/-}$) are more susceptible to the inflammatory response of endotoxemic shock than WT mice with both copies of the endogenous *AM* gene.

Elevated serum TNF-α levels in AM$^{+/-}$ septic mice

Serum TNF-α was quantified using ELISA before and during LPS challenge in WT and *AM*$^{+/-}$ mice. Basal TNF-α levels prior to LPS treatment were similar in both genotypes (30 pg/ml +/- 5.59 for *AM*$^{+/-}$ versus 19.41 pg/ml +/- 4.69 for WT, p=0.174, Fig.5.2). Both WT and *AM*$^{+/-}$ mice had a marked elevation in TNF-α 1 hour after LPS administration. However, *AM*$^{+/-}$ mice showed a more robust response and secreted more than two-fold higher TNF-α than WT mice within the first hour of LPS challenge (598.83 pg/ml +/- 59 for *AM*$^{+/-}$ versus 268.18 pg/ml +/- 41 for WT, p=0.0004, Fig.5.2). These results are consistent with our experiments measuring TNF-α gene expression and provide further evidence that *AM*$^{+/-}$ mice are more susceptible to LPS-induced inflammation than WT controls.

Increased ALT and AST activity in AM$^{+/-}$ septic mice

Liver damage is a hallmark characteristic of endotoxin-induced septic shock and can be assessed by liver enzyme activity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are thus accurate indicators of liver cell damage

and inflammation (23). In untreated mice of both genotypes, serum ALT levels were similar (11 U/L +/- 2.3 for *AM*$^{+/-}$ versus 9.73 U/L +/- 1.06 for WT, p=0.63). Following a 24-hour, 100 mg/kg LPS challenge, *AM*$^{+/-}$ mice showed significantly higher ALT activity than WT controls (528.1 U/L +/- 158 for *AM*$^{+/-}$ versus 69.4 U/L +/- 15.6 for WT, p=0.02). Similarly, basal serum AST activity was not significantly different between WT and *AM*$^{+/-}$ mice (105.7 U/L +/- 7.5 for *AM*$^{+/-}$ versus 111.8 U/L +/- 6.1 for WT, p=0.56). However, after 24 hours of LPS challenge, *AM*$^{+/-}$ mice exhibited significantly higher AST activity (878 U/L +/- 181 for *AM*$^{+/-}$ versus 283.1 U/L +/- 45.5 for WT, p=0.01) (Fig. 5.3). Perhaps due to the isogenic 129S6/SvEv strain used in these studies, histological analysis and myeloperoxidase assays for neutrophil infiltration revealed no significant differences between LPS-challenged livers of WT and *AM*$^{+/-}$ mice. Nevertheless, these data clearly demonstrate that reduction of *AM* gene expression by 50% in *AM*$^{+/-}$ mice leads to more severe end-organ damage after septic shock than in mice with 2 copies of the *AM* gene.

Dynamic regulation of AM receptor genes in AM$^{+/-}$ septic mice

To determine whether the expression of the *AM* gene affects homeostatic alterations in the expression of genes encoding for AM receptors (*Calcrl*, *Ramp2*, and *Ramp3*) during sepsis we used quantitative RT-PCR of total lung RNA from septic WT and *AM*$^{+/-}$ mice. We found that *Ramp2* and *Calcrl* expression levels were markedly reduced 97.3% and 98.1%, respectively, while *Ramp3* expression was elevated 23.8 fold in WT mice treated with LPS. Importantly, similar changes in gene expression were observed in *AM*$^{+/-}$ mice; *Ramp2* expression was reduced

92.8%, *Calcrl* was reduced 95% and *Ramp3* was elevated 24.9 fold (Fig. 5.4). In addition, we also measured expression levels of another putative AM receptor, called L1, in WT and *AM*$^{+/-}$ septic mice, and found the expression level of this gene to be reduced in LPS-treated mice of both genotypes (75.7% reduction in WT mice and 68% reduction in *AM*$^{+/-}$ mice, data not shown). Taken together, our results support the concept that robust and dynamic changes in the expression of genes responsible for mediating AM signaling occur during septic shock. Moreover, since the magnitude of these changes was not different between WT and *AM*$^{+/-}$ mice, we conclude that the level of AM peptide is not primarily involved in mediating responsiveness to AM signaling at the level of receptor expression.

Discussion

Many in vitro studies have suggested an important regulatory role for AM in sepsis and other inflammatory diseases (12, 17, 18, 27, 30, 32). Moreover, genetic over-expression of AM in the mouse vasculature or therapeutic administration of AM peptide in rats or mice had beneficial effects on reducing the inflammatory and hemodynamic insults elicited in septic shock (25). However, whether genetic reduction of AM can cause a more severe response to sepsis has not yet been determined. The present study was designed to test the effects of a genetic reduction in AM in a murine model of LPS-induced septic shock.

We found that the administration of LPS induced a more severe inflammatory response in *AM*$^{+/-}$ mice than in WT LPS-treated mice. Regulation of the inflammatory response by AM occured at both transcriptional and translational

levels. The expression of two pro-inflammatory cytokines, *TNF-α* and *IL-1β*, was significantly higher in *AM*$^{+/-}$ septic mice than in WT septic mice. In addition, serum TNF-α levels were more than 2-fold higher in *AM*$^{+/-}$ septic mice than in WT septic mice. This data is consistent with previous reports showing that AM reduces the production of TNF-α in macrophages and Kupffer cells (30).

In addition to increased cytokine production, there was also a significant elevation in ALT and AST enzyme activity in *AM*$^{+/-}$ septic mice, when compared to WT septic mice. These results are in agreement with a report by Shindo et al. showing that mice overexpressing AM in their vasculature were resistant to LPS-induced liver damage (25).

Notably, we failed to observe any differences in end-organ histology (liver, kidney), pulmonary edema formation or overall survival of *AM*$^{+/-}$ septic mice compared to control septic mice at all doses and times examined. We therefore conclude that a modest genetic reduction in *AM* to 50% of WT levels does not significantly impact on the pathological morbidity and mortality of septic shock. Nevertheless, it is likely that the robust induction of *AM* expression and the modulation of its receptors plays an important role in mediating the septic response.

Recent studies by others revealed a dynamic change in the expression of genes involved in AM signaling during sepsis. Nagoshi et al. found that TNF-α significantly reduced the expression of *Calcrl*, *Ramp1* and *Ramp2* in cultured human coronary artery smooth muscle cells in a time and dose-dependent manner (19). Furthermore, Ono et al. examined the expression of *Calcrl* and *Ramp1*, *2*, and *3* in several tissues from LPS-induced septic mice. *Calcrl* and *Ramp2* expression were

significantly decreased in lungs of septic mice, whereas *Ramp3* message levels were increased approximately 40 fold in lungs after 12 hours of LPS challenge (21). Using a polymicrobial model of sepsis, Ornan et al also showed that *Ramp3* expression is elevated in lungs during the early hyperdynamic stage of sepsis, but not in the later hypodynamic phase (22). Similar to these studies, we also found that LPS induced a significant change in the expression patterns of genes involved in AM signaling, characterized by a "switch" from *Ramp2* to *Ramp3* expression in the lungs of septic animals. Importantly, we further established that genetic reduction of *AM* does not impact on the magnitude of these changes, since the response to LPS was similar in $AM^{+/-}$ and WT mice. Although some studies have suggested that modulation of *Ramp2* and *Ramp3* may act to alter the clearance of AM peptide, our data show that the absolute level of AM peptide does not directly impact on their expression.

We conclude that during septic shock the dynamic modulation of AM and its receptors primarily functions to dampen the inflammatory response. Therefore, our in vivo studies using mice with genetically reduced levels of AM support the use of AM therapy to help counteract the detrimental effects of inflammation during septic shock in humans.

Figure 5.1

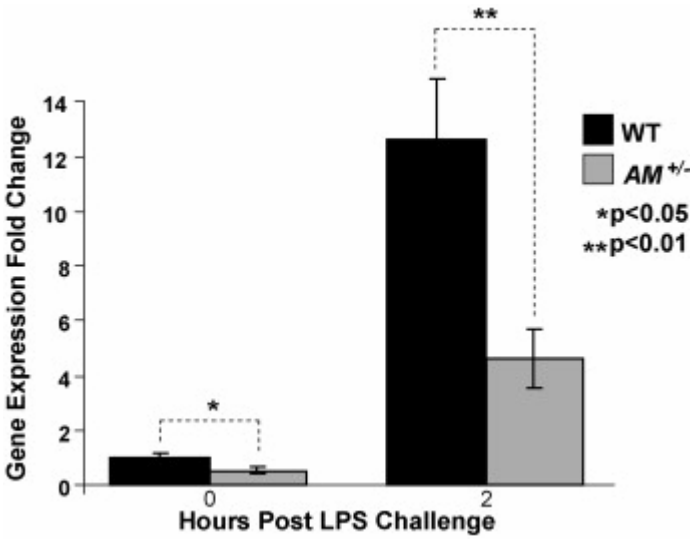

Fig. 5.1. *AM* gene expression levels before and after LPS challenge. Total lung RNA was prepared from WT and *AM*$^{+/-}$ mice treated with or without LPS for 2 h. *AM* gene expression was measured by quantitative RT-PCR. At least seven animals were used in each group.

Figure 5.2

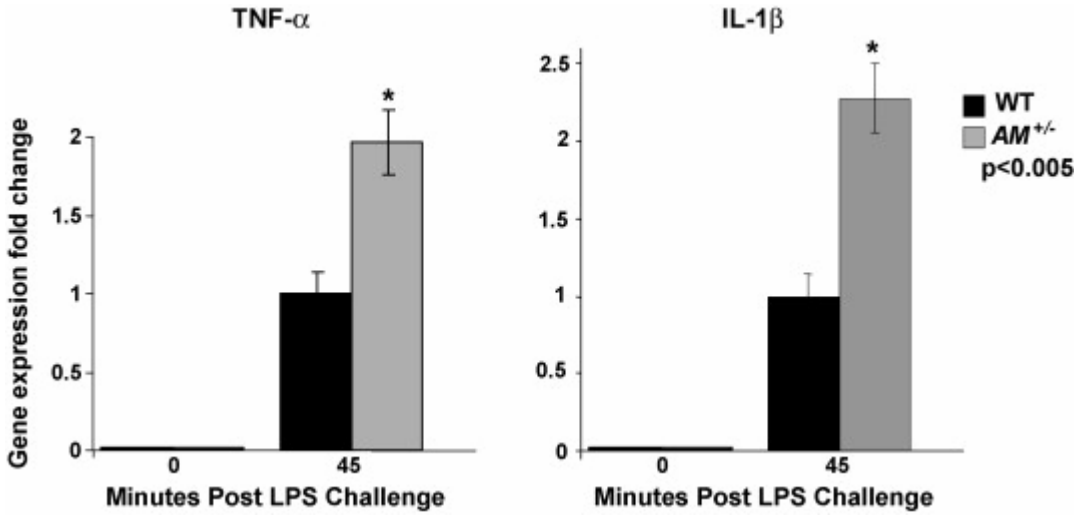

Fig. 5.2. Gene expression levels of *TNF-α* and *IL-1β* following LPS challenge. Total lung RNA was prepared from WT and *AM*$^{+/-}$ mice treated with or without LPS for 45 min. Expression of these genes was measured by quantitative RT-PCR. Four mice were used in each group.

Figure 5.3

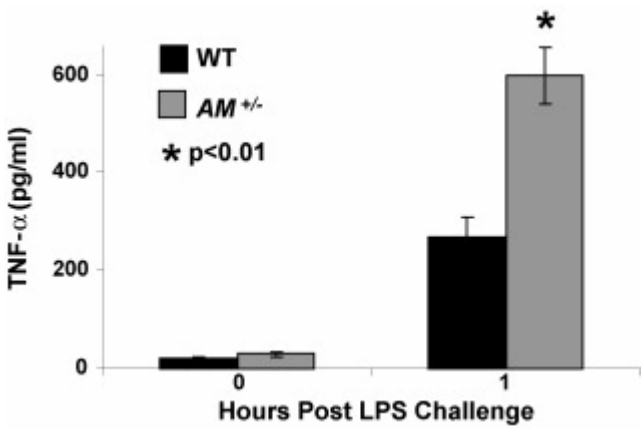

Fig. 5.3. Serum TNF-α levels following LPS challenge. Serum from WT and *AM*$^{+/-}$ mice was collected by tail-vein bleed at the indicated time points following treatment with 100 mg/kg LPS. TNF-α was analyzed by ELISA. *n* = 8 for WT mice and *n* = 9 for *AM*$^{+/-}$ mice.

Figure 5.4

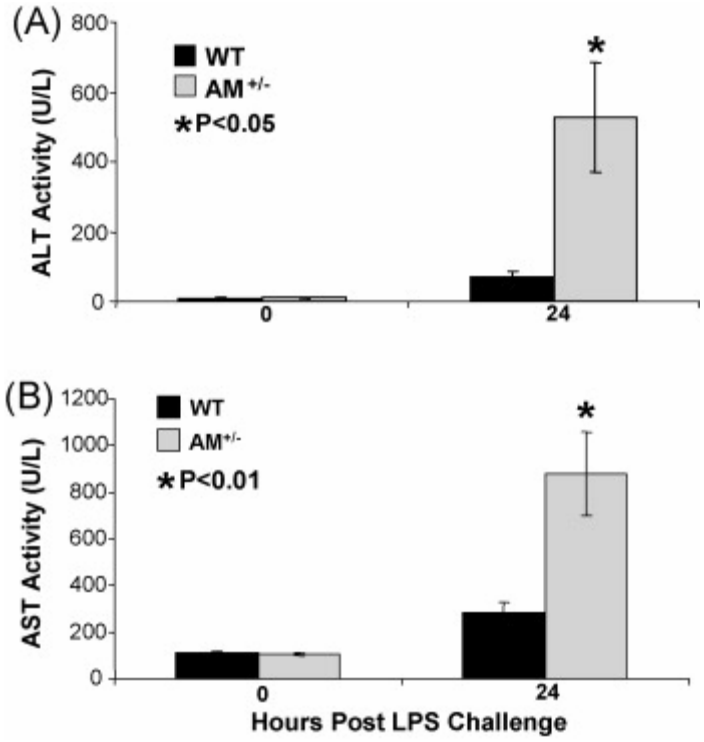

Fig. 5.4. Serum (A) ALT and (B) AST levels following LPS challenge. Serum from WT and *AM*$^{+/-}$ mice treated with or without LPS for 24 h was collected. At least six mice were used in each group.

Figure 5.5

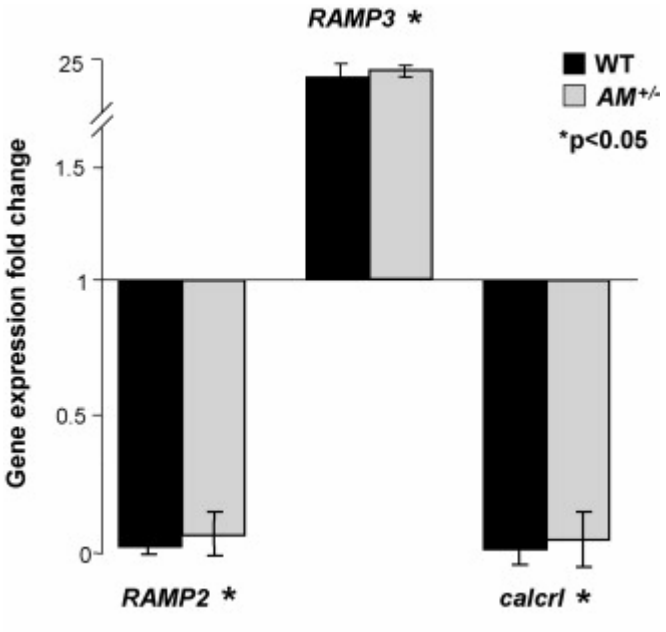

Fig. 5.5. AM receptor gene expression in WT and *AM*$^{+/-}$ mice following LPS challenge. Total lung RNA was prepared from WT and *AM*$^{+/-}$ mice treated with or without LPS for 12 h. Expression of *Ramp2*, *Ramp3* and *Calcrl* were measured by quantitative RT-PCR. Four mice were used in each group.

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CHAPTER 6

CONCLUSIONS

This thesis describes the use of animal models to examine the *in vivo* role of adrenomedullin signaling (AM, CLR, RAMP2 and RAMP3) in different physiological contexts including embryonic development, cardiovascular disease and sepsis. Since multiple animal models were used to study a variety of biological processes, numerous independent conclusions can be made which collectively enhance our current understanding of AM signaling and its *in vivo* physiological actions. Key points and potential future directions for each chapter are outlined below.

Chapter 2

Key Points

• Genetic deletion of *Calcrl* leads to mid-gestational embryonic lethality with severe generalized edema and cardiovascular defects.

• These defects almost identically phenocopy those previously observed in *AM* knockout mice (5), providing the first *in vivo* genetic evidence that CLR is the primary AM receptor.

Future Directions

Although *Calcrl* embryos die *in utero* with multiple defects, this model still holds much potential for helping us better understand the role of CLR in embryonic development and cellular function. Although technically challenging, echocardiography can be used to assess blood flow, cardiac function and overall development of *in utero* mouse embryos (20). Perhaps this state-of-the art technology could allow us to observe embryonic development over the course of a few days (i.e. from E10.5-E12.5) and provide insights beyond those gleaned from

traditional phenotyping methods. Also, a variety of cell types that completely lack *Calcrl* can be generated from knockout embryos. Fibroblasts, cardiomyocytes and endothelial cells are some of the culturable cell types that could be used from these mice to study different biological parameters. For example, endothelial cell cultures could be used to test the role of CLR in regulating permeability and may help resolve the never-ending debate of why *Calcrl* knockouts develop such severe edema. Of course, we must not forget that *Calcrl* heterozygote mice are also available. Since these mice have no obvious defects, they can be used in a variety of physiological studies to examine the role of CLR in heart disease, sepsis and kidney function, just to name a few. Additionally, *Calcrl*$^{+/-}$ mice can be crossed to other mouse models to study the effects of different mutations on a "sensitized" *Calcrl* genetic background.

Chapter 3

Key Points

• Because global *Calcrl* knockouts die *in utero* with multiple developmental defects, it was necessary to study the role of CLR in specific cell types. Thus, we generated mice with a "floxed" *Calcrl* allele, which could be crossed to any cell-specific Cre recombinase mouse line.

• Floxed *Calcrl* mice were crossed to α-MHCCre mice (1) to disrupt *Calcrl* expression specifically in cardiomyocytes, while retaining normal expression levels in all other cell types.

• Cardiomyocyte-specific *Calcrl* knockouts are born at the expected Mendelian ratios and survive into adulthood with no obvious defects. Thus, loss of *Calcrl* in

cardiomyocytes is not the cause of edema or embryonic lethality observed in the global knockouts (chapter 2).

• Cardiomyocyte-specific *Calcrl* knockouts were tested in two different heart disease models – transverse aortic constriction and angiotensin II infusion. In both models, development of cardiac hypertrophy and fibrosis was evident, but there were no significant differences between control and knockout animals.

• No significant differences in cardiomyocyte contractility were observed between control and knockout cardiomyocytes.

Future Directions

The generation and characterization of cardiomyocyte-specific *Calcrl* knockouts answered a burning question in the AM field: does loss of *Calcrl* expression in the myocardium contribute to the generalized edema and/or embryonic lethality? Since it does not, we are now also crossing the floxed *Calcrl* mice to other Cre lines to study the role of CLR in other cell types. In fact, our lab has already crossed the floxed mice to the Tie2Cre line (12) to delete *Calcrl* specifically in endothelial cells. As recently reported (JCI, article in press) these endothelial-specific *Calcrl* knockouts die at mid-gestation from severe generalized edema, strikingly similar to the original global *Calcrl* knockouts. Thus, AM signaling through CLR in endothelial cells is necessary for normal embryonic development and survival.

It would be interesting to generate a transgenic mouse line that **over-expresses** *Calcrl* in endothelial cells and cross it to global *Calcrl* heterozygotes to ultimately produce mice that lack *Calcrl* in all cells except endothelial cells. Such a

strategy would allow us to address whether *Calcrl* expression in endothelial cells is sufficient to maintain normal embryonic development. Other members of the lab are currently crossing floxed *Calcrl* mice to the SM-22Cre line which is specifically expressed in smooth muscle cells (11). Results from these crosses are yet to be determined.

Another interesting aspect of this study is the fact that loss of *Calcrl* in the myocardium had no effect on the heart's response to aortic constriction or angiotensin II infusion. This is surprising, since $AM^{+/-}$ mice suffer more severe cardiac hypertrophy and fibrosis than wild-type controls in both of these models (16, 17). One possibility is that the role of CLR in the vasculature, rather than the myocardium, is more important in regulating the heart's response to stress. This hypothesis can be tested with a couple of different mouse lines. First, assuming that the smooth muscle cell-specific *Calcrl* knockouts survive into adulthood with no inherent defects, they could be challenged with both the aortic constriction and angiotensin II infusion disease models. Secondly, an inducible endothelial cell-specific Cre line (VE cadherin) could also be used to examine the endothelial-specific role of CLR in regulating the cardiac response to stress.

Chapter 4

Key Points

• Generation of *Ramp2* and *Ramp3* knockout mice is described.

• *Ramp2* knockouts die *in utero* at ~E14.5, while *Ramp3* knockouts are born at the expected Mendelian ratios and, except for reduced body mass at old age, have no obvious phenotypic defects.

• While several reports have consistently demonstrated that both RAMP2 and RAMP3 interact with CLR to confer an AM receptor (2, 3, 8-10, 14, 18), our study suggests that RAMP2 is the primary RAMP involved in AM signaling, at least during embryonic development. Further studies are required to determine the *in vivo* role of RAMP3.

Future Directions

Several future directions stem from this study. Since *Ramp2* knockouts are embryonic lethal the most obvious direction is to generate a floxed *Ramp2* allele that could be deleted in a cell-type-specific manner. Another interesting mouse model would be one in which the coding sequence for *Ramp2* is replaced with the coding sequence for *Ramp3*. Thus, in addition to expressing *Ramp3* in its normal temporal and spatial expression pattern, this model would also express *Ramp3* when and where *Ramp2* are normally expressed. The main question would be whether or not *Ramp3* can rescue the embryonic lethality that results from a loss of *Ramp2*. This experiment would provide insight into how the different RAMPs are utilized in AM signaling and may reveal significant functional differences between them.

It is surprising that *Ramp3* knockout mice are almost completely normal. *Ramp3* expression is elevated in several conditions, including cardiovascular disease and sepsis (18, 19, 28). As stated in chapter 5, *Ramp3* expression is elevated almost 30 fold in lungs of mice that were treated with LPS. Pilot studies

from our lab showed that *Ramp3*$^{-/-}$ mice respond similarly (inflammation and survival) to wild-type controls when challenged with LPS. Future studies directed at why *Ramp3* is so robustly elevated in response to LPS would be interesting. Is the *Ramp3* transcriptional response dependent on NF-κB and/or cytokines? Determining which cell-types (i.e. macrophages, neutrophils, endothelial cells) *Ramp3* is elevated in might provide clues to the function it serves in response to LPS or other inflammatory factors. Is vascular AM responsiveness altered when RAMP3 is absent? These questions and many more would help to fill a major gap in our understanding of the role RAMP3 plays in sepsis and inflammation.

Ramp3 expression is also significantly elevated in the myocardium in response to angiotensin II (15). To further study the role of RAMP3 in the cardiovascular response to angiotensin II we are currently crossing *Ramp3*$^{-/-}$ animals to mice that over-express renin (RenTgMK). These RenTgMK mice develop severe cardiac hypertrophy and fibrosis due to consistently high plasma renin and angiotensin II levels (4). The goal of this study is to determine if *Ramp3*$^{-/-}$ mice respond differently (i.e. cardiac and renal damage) to chronically high angiotensin II levels than wild-type controls treated in the same manner.

Chapter 5

Key Points

• Mice heterozygous for the *AM* gene (*AM*$^{+/-}$) were used to examine the role of AM in regulating the inflammatory response to LPS-induced septic shock.

• *AM*$^{+/-}$ mice experienced a much more severe inflammatory response, characterized by inflammatory cytokine levels and markers of liver damage, than wild-type control mice.

• The same *Ramp2*, *Ramp3* and *Calcrl* expression pattern which occurs following LPS challenge in wild-type mice also occurred in *AM*$^{+/-}$ mice, suggesting that the level of AM peptide during septic shock does not influence the transcriptional regulation of its receptor components.

Future Directions

Chapter 5 demonstrates the important role of AM in regulating the inflammatory response to LPS-induced septic shock. This study is supported by several other reports which describe AM as a potent anti-inflammatory factor as well as a key regulator of the hemodynamic response during septic shock (23, 25-27). For the most part, AM is beneficial in septic shock and is a likely therapeutic candidate for patients suffering from this condition. However, it is well-known that vascular responsiveness to AM is reduced during the later stages of sepsis, when multiple organ failure begins to occur (24). This change in AM responsiveness is probably due to the drastic reduction of *Ramp2* and *Calcrl* expression following prolonged exposure to LPS or infection. Therefore, in order for AM to be used as an effective therapeutic, we must find a way to maintain vascular responsiveness to AM throughout the course of sepsis.

Pentoxifylline (PTX) is an anti-inflammatory agent that has been shown to have several beneficial effects in sepsis, including maintenance of vascular AM responsiveness (13, 22). Whether or not PTX does this by solely acting to reduce

inflammatory cytokines is unclear. It is possible that PTX also alters AM signaling properties independently of its anti-inflammatory effects. A future study aimed at examining the effects of PTX on AM signaling components may help to enhance our understanding of the role AM plays in sepsis and how AM could be used clinically to treat patients.

Other future experiments could be designed to investigate the transcriptional regulation of *AM* following LPS exposure. Although it is well-known that *AM* is elevated in response to LPS and cytokines (7), little is known about how it is transcriptionally regulated at the promoter level. NF-κB and STAT3 are two transcription factors that are activated in response to LPS and may be involved in regulating *AM* transcription (6, 21).

Summary

Clearly, AM and its signaling partners are involved in a variety of biological activities. Much of our current understanding of AM signaling had previously come from *in vitro* studies or "non-genetic" animal models. Using modern methods in gene targeting, I have presented some of the first *in vivo* genetic evidence which supports many of those earlier studies. Additionally, novel concepts were introduced which will enhance our understanding of AM biology and direct the course of future studies.

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