Published in final edited form as:

Circ Res. 2018 January 19; 122(2): 319–336. doi:10.1161/CIRCRESAHA.117.310978.

Multifunctional Role of Chymase in Acute and Chronic Tissue Injury and Remodeling

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Abstract

Chymase is the most efficient angiotensin II-forming enzyme in the human body and has been implicated in a wide variety of human diseases that also implicate its many other protease actions. Largely thought to be the product of mast cells, the identification of other cellular sources including cardiac fibroblasts and vascular endothelial cells demonstrates a more widely dispersed production and distribution system in various tissues. Furthermore, newly emerging evidence for its intracellular presence in cardiomyocytes and smooth muscle cells opens an entirely new compartment of chymase-mediated actions that were previously thought to be limited to the extracellular space. This review illustrates how these multiple chymase-mediated mechanisms of action can explain the "residual risk" in clinical trials of cardiovascular disease using conventional renin-angiotensin system blockade.

Keywords

myocardial infarction; diabetes; atherosclerosis; ischemia reperfusion injury; heart failure

Introduction

The destructive and protective functions of mast cell proteases have led to the labeling of the mast cell as a *contextual chameleon.*[1] The major protease contents of the mast cell include tryptase, chymase, carboxypeptidase A, and dipeptidyl peptidase I (DPP I). The widely diverse actions of mast cells are determined by the dynamic changes in mast cell contents that is dependent on the type of stress and resulting local environment.[1–5] Of these mast cell products, the serine protease chymase (E.C. 3.4.21.39) has received a great deal of attention

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Conflicts of Interest: None.

over the past 30 years. This ~30 kD protein is the predominant angiotensin II (Ang II)-forming enzyme in the human heart and has a catalytic efficiency 20-fold greater than that of angiotensin converting enzyme (ACE).[6,7] Although this function is central to the role of renin angiotensin system (RAS) blockade in the treatment of cardiovascular disease, the translation of chymase inhibition to the bedside has been lacking. One reason is the misleading notion that chymase inhibition may be unnecessary because Ang II type I (AT_1) receptor blockers will negate any excess Ang II formation that escapes ACE inhibition. The second reason stems from a reluctance to interpret the limited efficacy of RAS inhibitors in major clinical trials as a failure of these therapies to directly access the intracellular sites at which Ang II is formed.[8,9]

Recent reviews have addressed the question of "residual risk" of ACE inhibitors, AT_1 receptor blockade, or their combinational use in the many stroke, hypertension, diabetes, and heart failure trials reported over the past 20 years.[8–12] The discussion of these analyses has largely focused on the complete suppression of Ang II formation or the production of other functionally active Ang peptide derivatives.[8,9] The need for new therapeutic targets is now fueled by the failure of dual RAS blockade with the addition of the direct renin inhibitor aliskiren in patients with chronic heart failure[13,14] or with type II diabetes.[15] Likewise, the success of neprilysin blockade added to AT_1 receptor blockade in patients with heart failure demonstrates only a 20% benefit over a ACE inhibitor-based treatment approach.[16,17]

This review will highlight the extensive evidence implicating chymase in pathobiology, with an emphasis on: 1) human and animal studies demonstrating the multifunctional roles of chymase in tissue injury and remodeling, 2) clinical applications of chymase inhibition that complement ACE inhibitor and/or AT_1 receptor blockade, and 3) intracellular location and alternative cellular sources of chymase in addition to mast cells.

Multifunctional Mechanisms of Chymase in Tissue Remodeling

Besides being the most efficient Ang II-forming enzyme, chymase has an amazing number of roles in tissue remodeling that are mediated by its direct protease actions, such as:

1. *cleavage of matricellular proteins and peptides*, including laminin and fibronectin important in cell survival;[18–20] gap junction proteins essential to regulation of intestinal permeability;[21] and insulin-derived growth factor 1 (IGF-1), which negates the beneficial effects of IGF-1 in ischemia/reperfusion injury;[22] and
2. *activation of peptide and enzyme precursors,* including matrix metalloproteinases (MMPs) such as MMP-9;[23–26] transforming growth factor-β (TGF-β);[27–29] stem cell factor (SCF);[30] kallikrein, which produces bradykinin (BK) and causes further mast cell degranulation and chemotaxis;[31,32] Interleukin-6 (IL-6) and IL-1β;[33] pre-proendothelin I (pre-proET1);[34] and IL-18, which is involved the pathophysiology of atopic dermatitis.[35]

Chymase in Human Pathobiology

The ubiquitous nature and multiple functions of chymase explain why the literature is replete with the importance of mast cells and chymase in various human organ pathologies.

Table 1 provides a list of the many disease pathologies that represent a partial list of reports of chymase in human disease.[1–5] Many underscore the mechanistic role of chymase in MMP activation in atherosclerosis,[36–47] vulnerability of the atherosclerotic plaque and aneurysm formation,[48–50] and angiogenesis in tumor progression. [51–53] In concert with its major role as an Ang II forming enzyme in the human heart,[6,7,54] chymase has been associated with TGF-β activation and ET-1 formation from pre-proET-1 in pulmonary fibrosis[55,56] and chronic obstructive pulmonary disease,[57] chronic kidney disease,[58,59] polycystic kidney disease,[60] diabetic nephropathy[61–63] and retinopathy,[64] kidney transplant rejection,[65] and keloid formation in the skin.[66,67] With regard to its direct protease actions, chymase has been implicated in the direct breakdown of placental matricellular proteins and vascular permeability in preeclampsia,[68–70] Crohn's disease,[71] and atopic dermatitis.[72] Finally, a number of studies have demonstrated chymase-mediated vasoconstriction in isolated human internal mammary arteries, coronary arteries, and saphenous veins (Figure 2).[73–77]

Altogether, chymase has a pervasive prese**n**ce in human organ systems, with direct and indirect protease actions that mediate acute tissue injury and chronic remodeling. However, the net effect of chymase in tissue remodeling is best exemplified by the widely divergent effects of its presence in mediating extracellular matrix (ECM) breakdown in atopic dermatitis and excessive ECM formation in skin keloids. As will be emphasized in this review, the ultimate phenotype of chymase activation depends on the nature and timing of the stress and the prevailing milieu. In ECM homeostasis, cellular and tissue remodeling, chymase is at the epicenter of a critical balance of: (1) pro-fibrotic ET-1 and Ang II formation, myofibroblast formation, and TGF-β activation, (2) the anti-fibrotic protease activation of MMPs, direct digestion of matricellular proteins, and formation of BK, and (3) inflammatory cytokines and SCF activation and chemotaxis of inflammatory cells (Figure 1).

Chymase in Preclinical Animal Models

Transgenic Mice—The problem with addressing the role of chymase in preclinical animal studies is that the mouse and rat have multiple isoforms of chymase that forms and degrades Ang II (β-chymase). Because of these diverse catalytic specificities, the clinical translatability of pharmacologic blockade cannot be guaranteed, as humans have only the α-chymase isoform.[78,79] Nevertheless, the mouse mast cell protease-4 (MCP-4) is the isoform most comparable to human α-chymase because it has similar substrate specificity, tissue distribution, serglycin proteoglycan-binding properties, and the ability to convert Ang I to Ang II.[78] It is of interest that the MCP-4-knockout mouse at baseline defines the importance of a functional chymase in mediating MMP activation and ECM homeostasis.[80,81] In these mice, histological analysis reveals an increase in collagen in lung and ear tissue and a marked increase in fibronectin. Thus, at baseline there appears to be a net increase of ECM production that results from the reduction of chymase activation of MMP-2 and -9 and a decrease in chymase-mediated degradation of fibronectin.

Accordingly, acute tissue injury that is largely mediated by MMP activation, matricellular breakdown, and inflammatory cell infiltration is ameliorated in MCP-4-knockout mice. Knockout of MCP-4 also improves left ventricular (LV) infarct size and LV dilatation and

function after cardiac ischemia/reperfusion injury.[22] Moreover, inflammatory cell infiltration and MMP and TGF-β activation are significantly decreased resulting in reduced fibrosis from bleomycin-induced lung injury.[82] MCP-4 knockout also leads to decreased blister formation in a transgenic model of bullous pemphigoid,[83] protection against ulceration and scarring after a scald dermal burn injury,[84] and attenuation of aortic aneurysm formation in a model of elastase perfusion of the aorta.[85] Other studies in MCP-4-knockout mice demonstrate decreased conversion of pre-proET1 to ET-1 and the reduction of pulmonary fibrosis in response to bleomycin injury[86] and atherosclerosis.[87] Ulceration and subsequent scarring from burn injury have also been linked to MCP-4 digestion of the tight junction protein claudin,[88] which is prevented in MCP-4-knockout mice but restored by addition of chymase to injured skin. Knockout of MCP-4 also affords protection in mouse models of immune complex-induced encephalomyelitis,[89] arthritis,[90] and glomerulonephritis.[91]

However, there are equally important protease actions of MCP-4 that provide protection from inflammation through degradation of inflammatory cytokines in models of sepsis,[92] acute spinal cord[93] and post-traumatic brain[94] injury, and allergic airway disease,[95,96] or in the breakdown of snake venom.[97] It is of interest that knockout of MCP-4 prevents the degradation of fibronectin and decreases the homing of inflammatory cells and fibrosis in a 7-day model of ureteral obstruction.[98]

Conversely, transgenic mouse models expressing human chymase are reported to have a three-fold increase in LV Ang II levels,[99] in addition to the presence of LV hypertrophy, mild hypertension,[100] and increased MMP-9 activity and IL-6 levels. It is of interest that human chymase transgenic mice have leukocytosis and emaciation, with a reduction in skin and visceral fat, and oligotrichia as well.[100] Moreover, streptozotocin-induced diabetes is associated with higher plasma glucose levels and a reduced survival rate in these transgenic mice.[101] A unique rat smooth muscle chymase, rat vascular chymase (RVCH), has been isolated from the spontaneously hypertensive rat (SHR).[102] Transgenic mice with conditional expression of RVCH targeted to smooth muscle cells have hypertension, with a hypertensive arteriopathy manifested by medial thickening in the mesenteric and pulmonary arteries and an increased vasoconstrictor response to phenylephrine.[103] Although RVCH has 80% homology to rat mast cell proteases I and II, it has a substrate specificity and *K*m and *K*cat values similar to those of the human chymase (α-chymase).

Thus, it is abundantly clear that human chymase and murine MCP-4, apart from Ang II forming capacity, mediate acute and subacute inflammatory insults that involve the multiple mechanisms of action outlined in Figure 1. In considering the translational implications of studies in mice, MCP-4-knockout mice have a baseline status of ECM production, while the transgenic mice overexpressing human chymase have a generalized emaciated appearance with evidence of systemic inflammation. Taken together, the *in vivo* phenotypes must be reviewed in the context of the nature of the acute stress, target organ, and timing after the acute stress. Many more pharmacologic studies in the next sections are conducted over a longer period of time and further demonstrate the many functions of chymase in tissue remodeling.

Pharmacologic Studies in Rodents—Although rats and mice have a collection of β-chymases that degrade Ang II,[78,79] a number of studies in these animals document the beneficial effects of chymase inhibitors in a wide variety of conditions that again highlight the many destructive protease actions of chymase (Table 2).[104–110] There are many more pharmacologic studies that evaluate chymase inhibitors on organ function and remodeling in the more clinically relevant hamster, which has an α-chymase with substrate specificity similar to that of the human chymase and lacks the β-chymases.[78,79,112–115] These studies demonstrate a number of beneficial effects of prolonged chymase inhibition in models of more chronic tissue remodeling and function (Table 2).[116–128] Chymase-mediated activation of SCF has an important role in the accumulation of mast cells, and these studies also demonstrated a decrease in the number of mast cells and other inflammatory cells with chymase inhibition[130] and, importantly, functional stabilization of the mast cell membrane.[131] The transgenic studies of MCP-4-knockout mice and the pharmacological studies in mice, rats, and particularly hamsters clearly show the multifunctional role of chymase in the pathophysiology of acute and chronic tissue injury and remodeling.

Pharmacologic Studies in Dogs—The dog provides one of the better animal models of human chymase biochemistry and physiology, in addition to the baboon[132] and macaque.[129] These species have only α-chymase with Ang II-forming capacity and MMP-activating potential similar to that of human chymase, without the potential of Ang II degradation of β-chymases.[78,79] In the dog, chymase inhibition attenuates neointimal hyperplasia 2 weeks after carotid balloon injury[133] and 4 weeks after grafted veins.[134] Prolonged chymase inhibition in dogs prevents progression of aortic abdominal aneurysm 8 weeks after elastase treatment,[135] development of fibrosis in the pacing tachycardia model of heart failure,[136] and development of ventricular arrhythmias after myocardial infarction.[137] The next section delves further into the multiple protease actions of chymase in the stretch stimulus of mitral regurgitation (MR) and ischemia/reperfusion injury in the dog.

Potential Cardiovascular Clinical Applications for Chymase Inhibition

Volume Overload

Chymase has a critical role in the myocardial wall thinning and LV chamber dilation in a pure volume overload (VO) in humans with isolated MR,[138–140] in dogs with experimentally produced MR,[141–145] and in mice[146] and rats with aortocaval fistula (Figure 3).[147–152] Chymase activity is increased in the left atrium of MR patients,[54,153] in the LV of the MR dog,[141–143] and in LV of the aortocaval fistula mouse[146] and rat.[149–152] Although LV ACE activity and Ang II levels are upregulated in the VO heart, AT_1 receptor blockade or ACE inhibition exacerbates ECM loss and LV dilatation due to the anti-fibrotic effects of these drugs.[144,145] The myocardial response to a pure VO is distinct from the response to pressure overload in that, as opposed to ECM accumulation, there is extensive loss of interstitial collagen and breakdown of matricellular stabilizing proteins, resulting in disruption of the focal adhesion complex and cardiomyocyte elongation and thinning.[154–156] In addition to forming Ang II, chymase activates kallikrein and in part may be responsible for increased LV BK in a pure VO in the rat[149,150] and dog.[156] BK not only perpetuates collagen loss due to its anti-fibrotic effects but also promotes mast cell recruitment and degranulation,

resulting in increased chymase activity that is further accentuated by the increase in BK with ACE inhibition.[150,157] Chronic chymase inhibition in the dog with isolated MR decreases MMP activation and elevated LV BK levels, prevents fibronectin degradation, and restores the focal adhesion complex, resulting in improved cardiomyocyte shortening and LV systolic function.[156]

More recently, we have reported fibroblast production of chymase in the LV of hearts with chronic VO *in vivo* and in isolated adult rat cardiac fibroblasts subjected to cyclical stretch *in vitro*.[152] In addition, chymase mediates autophagic digestion of pro-collagen and fibronectin within cardiac fibroblasts after cyclical stretch *in vitro* and VO *in vivo*.[152] These results indicate that the more plentiful fibroblasts, not just the sparsely populated mast cells, account for the abundance of chymase in the heart. Moreover, this finding offers a new chymase-mediated mechanism of ECM loss—through stimulation of fibroblast autophagic digestion of matricellular proteins in the context of a pure VO.

Myocardial Infarction

Understanding the underlying pathophysiology and timing of chymase activation in the context of its many potential direct protease actions may be an important factor in attenuation of acute/subacute tissue injury that translates into improvement in LV remodeling (Figure 3) and a better long-term outcome. More recently, chymase has been identified as an important pharmacologic target in limiting cardiac ischemia/reperfusion injury in the dog[158] and pig.[159] Again, these models demonstrate a significant acute decrease in MMP-9 activation, a decrease in neutrophil and mast cell infiltration, preservation of fibronectin and laminin, protection of the focal adhesion complex, and a decrease in apoptosis. Furthermore, studies in the MCP-4 knockout mouse identifies IGF-1 as a proteolytic target for chymase.[22] All of these chymase-mediated actions result in decreased infarct size and improved LV function.

In the **Co**operative **N**ew **S**candinavian **En**alapril **Su**rvival **S**tudy II (CONSENSUS II), the ACE inhibitor enalapril started within 24 hours after myocardial infarction does not improve survival over 180 days;[160] whereas, treatment with captopril at 48 hours and 14 days after myocardial infarction significantly decreased LV dilation over 3 months to 1 year.[161,162] In patients dying of myocardial infarction, chymase activity is increased nearly three-fold in both the infarcted and the non-infarcted portions of the LV and represents 90% of Ang II-forming capacity compared to ACE.[163] In addition, the left atrium shows the greatest increase in chymase activity, which also suggests that hemodynamic factors such as increased wall stress may be an important stimulus for chymase production/activation.[163] In the dog during ischemia/reperfusion injury, *in vivo* cardiac microdialysis shows an increase in interstitial fluid chymase activity in the infarct and non-infarct regions of the heart, thereby supporting an early activation of chymase with acute myocardial infarction.[158]

Thus, early chymase inhibition coupled with later ACE inhibition may provide the best outcome in the first month after myocardial infarction. In the hamster model of total left anterior descending artery occlusion, chronic ACE inhibition combined with chymase inhibition (as opposed to chymase or ACE inhibition alone) significantly improves LV remodeling and function, cardiomyocyte hypertrophy, and cardiac fibrosis, as well as

survival 1 month after coronary occlusion (Figure 4).[157] This study also demonstrates a BK-mast cell interaction that triggers mast cell degranulation and an increase in interstitial fluid chymase activity. This effect is abrogated in the mast cell-deficient mouse or with BK type 2 receptor antagonism in wild-type mice.[157] This finding suggests a novel mechanism of "ACE escape" with regard to failure of ACE inhibitor therapy. More importantly, combined therapy with ACE and chymase inhibition attenuates the potential pro-inflammatory effects of BK attendant with ACE inhibition, along with the multiple chymase-mediated protease actions (Figure 1).[157] The results of this study raise the question of whether the benefits of chronic ACE inhibition in myocardial infarction may be limited by the ACE inhibitor–dependent chymase release into the cardiac interstitial fluid space.

One of the newest discoveries regarding chymase is its presence within cardiomyocytes after ischemia/reperfusion in the dog.[158] Figure 5 demonstrates a marked influx of chymase (red) and breakdown of desmin (green) in the cardiomyocyte after 1 hour of occlusion and 2 hours of reperfusion. This now raises many questions regarding the possible role of chymase in cardiomyocyte ischemia/reperfusion injury that may involve activation of intracellular MMPs that have been connected with cardiomyocyte myofibrillar degradation and injury.[164–166] Currently, a new chymase inhibitor, BAY1142524, is being tested for treating patients with LV dysfunction after myocardial infarction in a phase 2 clinical trial (NCT02452515 — clinicaltrials.gov).

Vulnerable Atherosclerotic Plaque

In the setting of non-ST elevation myocardial infarction, which is the predominant presentation in the U.S. today, plaque stability is the key focus and the therapeutic target in the follow-up of these patients (Figure 3). Much of the subsequent risk for myocardial infarction resides in coronary arteries without significant angiographic stenosis, highlighting the importance of the vulnerable plaque. In the **P**roviding **R**egional **O**bservations to **S**tudy **P**redictors of **E**vents in the **C**oronary **T**ree study (PROSPECT), the mean angiographic stenosis of lesions that subsequently produced ischemic events was 32%.[167] Over the past 30 years, much attention has been devoted to the identification of the prevalence and detection of the thin-cap fibroatheroma with consensus of opinion regarding its propensity to rupture.[168–171]

Increases in the number of mast cells and the levels of chymase[36–39] and MMPs[172] have been identified in the atherosclerotic shoulder region of the infarct-related artery in the human, implicating chymase in plaque rupture by activation of MMPs.[36–39,46] In addition, chymase has been implicated in plaque erosion by inducing apoptosis in endothelial cells through direct protease actions that degrade collagen.[173] Other studies have demonstrated that chymase-mediated degradation of vitronectin and fibronectin, necessary for maintenance of focal adhesions, results in subsequent inactivation of focal adhesion kinase and Akt and activation of caspase-8 and -9.[174,175] Several excellent reviews of these aspects of mast cell proteases and chymase provide a strong argument for chymase inhibition in patients presenting with acute coronary syndrome, expecially those who are at high risk for plaque rupture in the ensuing 6 months.[176–178] Finally, chymase may also be directly or

indirectly involved in intimal lipid deposition mediated through degradation of ECM[177] and in cholesterol efflux by proteolysis of high density lipoprotein 3.[179]

The identification of increased chymase in the infarct-related artery[36–39] and the multiple studies demonstrating a predominant vasoconstrictor response in the coronary artery[73–75] further support a role for chymase in the pathogenesis of acute myocardial infarction. Taken together, there is a strong case made by these studies in humans as well as preclinical animal models that chymase may be an important target in acute and chronic atherosclerotic vascular disease.

Diabetes

A connection between diabetes and mast cell chymase in the heart, kidney, and vasculature has been documented in humans and preclinical animal models.[180] Advanced glycation end products (AGEs) have been shown to induce chymase expression and a predominance of chymase-dependent Ang II generation (70%) over ACE-dependent generation in human vascular smooth muscle cells via receptor for advanced glycation endproduct (RAGE)-ERK1/2 MAP kinase–dependent mechanism.[61] Furthermore, a three-fold increase in chymase immunostaining is demonstrated in renal and coronary arteries in diabetic versus nondiabetic patients.[61] A marked increase in chymase, AGEs, RAGE, and phosphorylated ERK1/2 has also been detected in the diabetic coronary arterial wall, spanning the endothelium, media, and adventitia.[61] It is of interest that in addition to mast cells, vascular smooth muscle cells are also identified as a major site of increased chymase expression in the human diabetic kidney. Finally, expression of chymase in the glomerulus and tubulointerstitial areas correlates with the increase in blood pressure and the severity of proteinuria.[62]

The suggestion of a non-mast cell source of chymase is consistent with *in vitro* studies in human vascular smooth muscle cells demonstrating that high glucose increases both chymase and ACE mRNA.[63] In the rat vascular smooth muscle cell, high glucose causes an increase in chymase expression and a switch from ACE-dependent to chymase-dependent Ang II formation.[181] In addition to Ang II formation, studies in hamsters[119–121] and rats[182,183] have connected the beneficial effects of chymase inhibition to the attenuation of oxidative stress, TGF-β activation, and fibrosis in the kidney and heart in the streptozotocin-induced diabetes model. It is well accepted that diabetic nephropathy is a microvascular complication of type II diabetes mellitus. Notably, leptin receptor-deficient *db/db* mice exhibit a switch from ACE-dependent to chymase-dependent afferent arteriole vasoconstriction and Ang II formation.[184,185]

In addition to the effects in vascular smooth muscle cells, exogenous high glucose upregulates chymase production in isolated cardiomyocytes and fibroblasts.[186–188] This intracrine function further underscores the potential of intracellular chymase-mediated Ang II signaling that is minimally affected by ACE inhibitors and AT_1 receptor blockers acting predominantly on the cell surface. A caution for combined therapy of conventional RAS inhibition has been signaled by the results of the **On**going **T**elmisartan **A**lone and in Combination with **R**amipril **G**lobal **E**ndpoint **T**rial (ONTARGET), in which combined AT_1 receptor blockade and ACE inhibition associated with more adverse events, without an

increase in benefit compared with either monotherapy in patients with cardiovascular disease or diabetes mellitus.[189]

The next section will discuss a *de novo*, noncanonical intracellular Ang II-forming process that proceeds from angiotensinogen to Ang-(1-12) to Ang II via chymase. The intracellular Ang II-forming capacity of chymase, in addition to its other protease actions (Figure 1), provides a rationale for chymase inhibition in narrowing the residual risk in clinical trials of RAS blockade in diabetes without a deleterious decrease in blood pressure.

Intracellular location and multiple cellular sources of chymase other than mast cells

At this point, it must be stressed that studies of chymase-mediated Ang II formation and/or cleavage of proteins and peptides have been largely focused on the chymase actions in the extracellular space of organs and tissue. As alluded to above, using cardiac microdialysis *in vivo* in the dog has revealed a compartmentalization of Ang II formation, with Ang II formation mediated by chymase in the cardiac interstitium and by ACE in the intravascular space.[190] In the ACE-knockout mouse, tissue chymase activity is upregulated such that tissue Ang II levels are preserved in the heart, lung, and kidney while circulating Ang II levels are undetectable.[191] As alluded to in the previous section, this tissue compartmentalization of chymase has now been expanded to include its role in the intracrine RAS.[192–194]

Chymase-mediated Ang II formation in neonatal cardiomyocytes and fibroblasts in response to glucose stress,[186–188] production of chymase by fibroblasts in response to VO in the rat,[152] and chymase production in smooth muscle cells in the human with diabetes[62] also generate questions regarding the compartmentalization of chymase's direct protease actions and Ang II formation within the cell. Results from a growing number of studies suggest that angiotensin-(1-12) [Ang-(1-12)] serves as an alternate intracellular precursor for the generation of Ang II.[195–197195–204] In particular, this concept is supprted by the presence of Ang-(1-12) along with chymase in left atrial myocytes of patients undergoing a Cox Maze procedure for atrial fibrillation ablation.[54,204] In support of this contention, conversion of Ang-(1-12) to Ang II by chymase within the adult rat cardiomyocyte results in an increase in the duration of the action potential followed by the generation of early afterdepolarizations.[205] In these experiments, a chymase inhibitor dialyzed intracellularly with Ang-(1-12) prevented the effect of intracellular Ang-(1-12) delivery on the potassium currents.[205] Importantly, this study directly demonstrates for the first time that chymase has an intracellular function that is affected when the AT_1 receptor blocker is delivered with Ang-(1-12) intracellularly, suggesting that intracellular AT_1 receptors are required for the signaling.[205] It is important to note that in the human heart failure heart, endothelial cells and other interstitial cells are identified as a major source of chymase in addition to mast cells.[206] Taken together, these results suggest that mast cells, endothelial cells, and fibroblasts are a major source of chymase in the human heart and raise the provocative question of whether chymase is also produced within cardiomyocytes or is largely taken up via endocytosis.

Earlier studies have demonstrated increased Ang-(1-12) uptake in neonatal myocytes cultured from the spontaneously hypertensive rat.[198] We have recently demonstrated that chymase is taken up by mouse cardiac myocytes (HL-1 cells)[158] and adult rat cardiac fibroblasts[152] by a dynamin-mediated process. It is of interest that other serine proteases, including neutrophil elastase and cathepsin G, have been shown to enter cancer cells by clathrin-mediated endocytosis.[207] However, the actual uptake mechanism *in vivo* remains elusive and the *in vitro* addition of the active protease may not reliably recapitulate the *in vivo* condition. Two recent studies have reported the delivery of exosomes containing Ang II[208] and AT_1 receptors[209] into cells, thereby providing another potential mechanism of chymase trafficking directly into the cardiomyocyte cytoplasm.

With regard to cellular uptake of RAS components, it has been 46 years since radiolabeled Ang II injected into the circulation of the rat *in vivo* was found in the nucleus.[210] Subsequently, AT_1 and AT_2 receptors have been located in the nuclear membrane[211,212] and shown to produce angiotensinogen/renin mRNA and nitric oxide (NO),[213] respectively, in response to Ang II. Ang II has been shown to bind to chromatin[214] with a subsequent increase in RNA synthesis and gene transcription of angiotensinogen and renin.[215] Ang II and its AT_1 receptor in endo-lysosomes produce increased cytosolic Ca^{2+}.[216,217] AT_1 and AT_2 receptors have also been identified on the inner mitochondrial membrane, and when activated, regulate NO production and respiration in isolated mitochondria (Figure 6).[218,219] Intracellular microinjection of Ang II directly in single kidney proximal tubular cells increases [Ca^{2+}] levels in the presence of extracellular losartan, suggesting activation of intracellular AT_1 and AT_2 receptors.[220] Loading of renal mesangial cell nuclei with fluorescent NO prior to incubation with Ang II (1 nM) produces NO that is blocked by the non-specific AT_2 receptor antagonist PD-123319 but not the AT_1 receptor blocker losartan.[220]

Overall, these studies reveal the presence of AT_1 and AT_2 receptors on various cellular organelles with documented secondary physiological and/or second messenger effects based on Ang II microinjection into intact cells.[220] Future work must unravel the intracrine from extracellular Ang II biology. The challenge will be to separate the similar intracellular and extracellular actions of Ang II, and will require studies that incorporate intracellular Ang II trafficking.[221–223]

Serine Protease Activation and Inhibition *in vivo*

Chymase is one of a large family of serine proteases in mast cells that have essential roles in blood coagulation, apoptosis, inflammation, and host immunity, among other processes.[1–5] Indeed, immune cells express a wide variety of serine proteases, such as granzymes in cytotoxic lymphocytes; elastase, proteinase 3, and cathepsin G in neutrophils; and chymase and tryptase in mast cells. All of these serine proteases have somewhat similar actions in tissue remodeling, as outlined in Figure 1. Although beyond the scope of this chymase review, additional therapeutic options in the innovative area of protease inhibition deserves some mention.

For example, the prominent role of neutrophils and Cathepsin G in ischemia reperfusion injury of the heart has led to the development of dual chymase-cathepsin G inhibitors.[224,225]

Administration of Cathepsin G-chymase inhibitor immediately after reperfusion of the ischemic myocardium demonstrates the acute (24 hour) and longer term effects (7 days) after 30 minutes of occlusion in mice treated daily after reperfusion.[226] At 24 hours dual inhibition significantly reduce the increase in pro-inflammatory signaling pathways, Signal Transducer And Activator Of Transcription 3ST and Nuclear Factor-κB, myeloperoxidase, and accumulation of pro-inflammatory cytokines TNF-α- and IL-1β. At 7 days, dual inhibition reduces myofibroblast density and accumulation of profibrotic connective tissue growth factor, and TGF-β gene expression that is associated with a decrease in interstitial collagen. Altogether, this results in a decrease in infarct area and significant attenuation of LV dilatation and improvement in LV function at 7 days. Collectively, these data underscore the importance of that dual inhibition of Cathepsin G and chymase in the early inflammatory influx and later extracellular matrix acculation in the ischemia reperfusion injury.

In addition to chymase, mast cell tryptase is present in the shoulder region of the vulnerable atherosclerotic plaque[36–38] and in abdominal aortic aneurysm.[227] A recent review on the status of currently available tryptase inhibitors reports newly available tryptase inhibitors (APC-366 and RWJ-58643) showing promise in clinical trials of psoriasis and ulcerative colitis.[228] Neutrophil elastase and mast cell tryptase are key proteases in mediating lung injury and remodeling in chronic obstructive lung disease, cystic fibrosis, and α1-antitrypsin deficiency. Neutrophil elastase is one of the main targets of α1-antitrypsin, an acute-phase reactant protein that functions primarily as a serine protease inhibitor.[229] The recent **R**andomized, placebo-controlled trial of augmentation therapy in **A**lpha 1-**P**roteinase **I**nhibitor **D**eficiency (RAPID) and the RAPID Extension trials confirmed the benefits of α1-antitrypsin therapy in slowing disease progression in patients with α1-antitrypsin deficiency.[230]

In the absence of a pathophysiologic stimulus, chymase and other serine proteases are held in check by members of the serpin superfamily, including α1-antitrypsin, α1-antichymotrypsin, and α2-macroglobulin, maintaining the balance of protease actions in tissue and in the circulation.[231,232] Providing support for this contention, we have used cardiac microdialysis to measure Ang II-forming capacity from the chymase-specific substrate [Pro^{11}-D-Ala^{12}] Ang I infused through a microdialysis probe into the dog heart *in vivo*.[158] At baseline, chymase-mediated Ang II formation is negligible, whereas Ang II production increases significantly after occlusion and reperfusion of the left anterior descending artery. Other studies have shown that chymase-mediated conversion of Ang I to Ang II is almost completely inhibited in human heart tissue extracts when incubated with skin interstitial fluid, which interestingly has high concentrations of α1-antitrypsin.[233] Numerous studies of mast cells in skin blister formation have emphasized the important *in vivo* interplay between serpins and mast cell chymase/tryptase in addition to the physiologic effects of heparin binding, which also inhibits the protease activities.[234] The identification of chymase production by cells other than mast cells raises questions regarding whether the same inhibitory packaging that is specific to mast cells is also in place in endothelial cells and fibroblasts when they are stimulated to produce chymase.

The importance of serpins *in vivo* has been demonstrated in transgenic mouse models of SERPINA3, the murine homologue of the human α1-antichymotrypsin. SERPINA3

overexpression promotes greater sarcolemma membrane integrity and stability in dystrophic mouse models and also promotes increased membrane residence of integrins.[235] SERPINA3N inhibits decorin degradation by granzyme B and leads to enhanced collagen remodeling and reinforcement of the adventitia that augments the rate of rupture and death in a mouse model of abdominal aortic aneurysm.[236] *In vitro* studies of smooth muscle cells plated on fibronectin demonstrated that in the absence of the proteinase inhibitors (α1-antitrypsin, α1-antichymotrypsin, and α2-macroglobulin), fibronectin is degraded and that promotes apoptosis and caspase activation that is prevented in the presence of inhibitors.[237] This interaction is further complicated *in vivo* by the fact that serpins may be degraded by proteinases such as MMPs[238–241] and also by chymase, which has a four-fold greater catalytic efficiency than that of MMPs in serpin degradation.[242] Thus, a chymase inhibitor may also preserve serpins indirectly by inhibiting MMPs and by directly preventing degradation of serpins.

On the other hand, cathepsin C, also known as DPP1, is a cysteine exopeptidase that is expressed in many inflammatory cells, but is especially abundant in mast cells.[1–3,243] DPP1 is clinically attractive because it is the upstream activator of tryptases, chymases, elastase, and cathepsin G, removing N-terminal pro-dipeptides from the zymogen forms of these proteases. The cathepsin C-knockout mouse lacks chymase activity in mast cells.[1–3,243] Although a substantial number of cathepsin C inhibitors have been developed, especially for their ability to decrease neutrophil-driven elastase activity in lung disease,[244] further clinical development has stalled due to challenges with drug design. In addition, there remains a question of whether cathepsin C protease activation in humans is as important as it appears to be in mice.[243]

Since mast cells contain large amounts of chymase and other proteases involved in tissue injury and adverse remodeling, there has been some interest in mast cell stabilizers in cardiovascular disease.[176,177,180] However, the bidirectional function of mast cells in mediating injury as well as host immunity is problematic for chronic implementation of mast cell stabilizers. Another potential concern is that chronic mast cell stabilization in chronic MR in the dog results in a decrease in LV systolic function that is further verified in isolated adult dog cardiomyocytes, where mast cell stabilization causes a marked depression of the cardiomyocyte calcium transient and fractional shortening.[245] As a result, inhibiting chymase or a combination of proteases is a more appropriate therapeutic approach. Such an approach is now especially enticing given that cells other than mast cells produce excessive chymase under pathologic stimuli such as hypoxia,[246,247] hemodynamic stretch,[152] and excessive glucose.[186–188] This finding, coupled with the known benefits of mast cell contents in host immunity and attenuation of inflammation,[1–3] in addition to the alternative cellular sources of chymase, may dampen the enthusiasm for mast cell stabilizers in humans with LV dysfunction.

Synopsis

As documented by this review, there is a plethora of evidence for chymase upregulation in cardiovascular disease in humans and for the beneficial effects of its blockade in preclinical animal models of myocardial infarction, ischemia reperfusion injury, cardiac VO, aortic

aneurysm formation, atherosclerosis, and diabetic kidney and cardiovascular disease (Figure 3). In addition, increased chymase expression has been reported in African Americans,[248] in aging rats,[249] in females versus males subjected to pressure overload,[250] and in oophorectomized rats.[251] Race, age, and gender add to the potential for chymase in explaining the "residual risk" associated with ACE inhibition and AT_1 receptor blockade in the treatment of cardiovascular disease. This concept, coupled with multifunctional protease actions of chymase and the multiple cellular sources of chymase other than mast cells, provide a strong rationale that chymase blockade will provide complementary or synergistic actions with current RAS and/or adrenergic blockade. This viewpoint is further fueled by emerging evidence for its intracellular presence and actions that are observed even in the presenceof ACE inhibition or AT_1 receptor blockade. The challenge for the future is to 1) further understand mechanisms of intracellular and extracellular chymase activation and how this may affect therapeutic efficacy in *acute* or *chronic* cardiovascular disease, 2) document synergism with conventional therapy in preclinical animal models, and finally, 3) translate this functional information into targeted clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Source of Funding: This work was supported by grants from Department of Veteran Affairs for Merit Review (Grant 1CX000993-01 and 1BX003664-01 to LJD) and NIH (Grant P01 HL051952 to CMF, JFC and LJD).

Non-standard Abbreviations and Acronyms

AGEs	advanced glycation end products
Ang II	angiotensin II
ACE	angiotensin converting enzyme
AT_1	angiotensin II type I
DPP I	dipeptidyl peptidase I
BK	bradykinin
ECM	extracellular matrix
IGF-1	insulin-derived growth factor 1
IL	Interleukin
LV	left ventricular
MCP-4	mast cell protease-4
MMPs	matrix metalloproteinases
MR	mitral regurgitation

NO	nitric oxide
pre-proET1	pre-proendothelin I
RVCH	rat vascular chymase
RAGE	receptor for advanced glycation endproduct
RAS	renin angiotensin system
SHR	spontaneously hypertensive rat
SCF	stem cell factor
TGF-β	transforming growth factor-β
VO	volume overload

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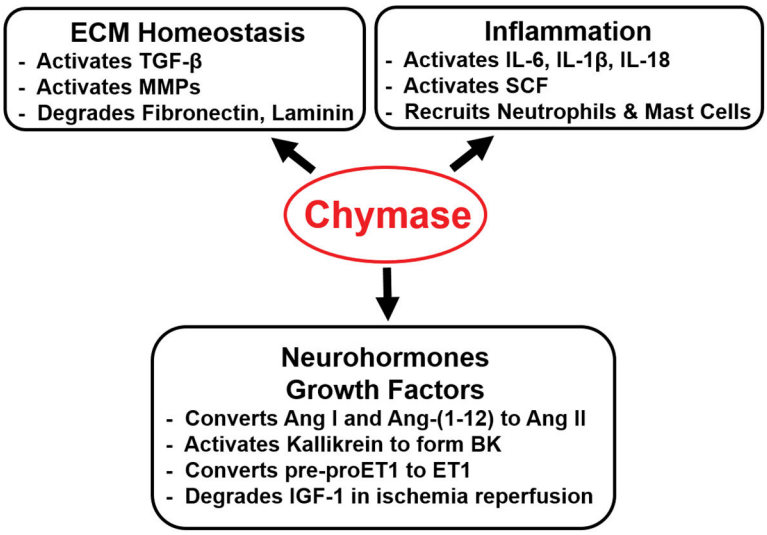

Figure 1.
Multifunctional Actions of Chymase in Acute and Chronic Tissue Injury and Remodeling.

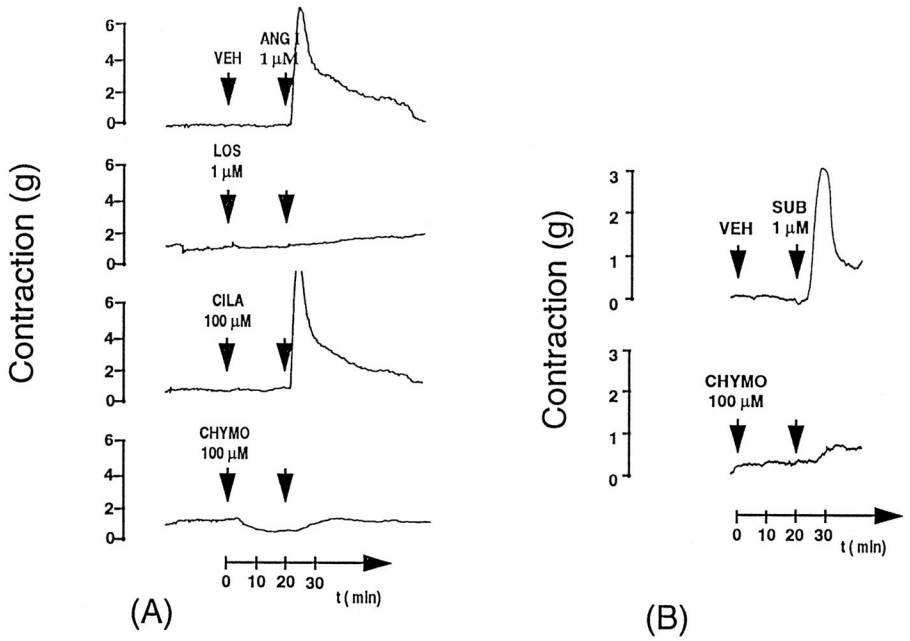

Figure 2. Original tracings of human coronary artery ring preparations from patients with heart failure

Panel A demonstrates the pressor response to 1 μmol/L Ang I or chymase-specific substrate [Pro11DAla12] Ang I (SUB) with cilazaprilat (CILA, 100 μmol/L), chymostatin (CHYMO, 100 μmol/L), chymostatin and cilazaprilat together, and losartan (LOS, 1 μmol/L). Arrowheads indicate the time of application. The inhibitors were added 20 minutes before the substrates. VEH indicates vehicle. Losartan and chymostatin completely blocked the pressor response to Ang I, whereas cilazaprilat was ineffective. As further confirmation of the chymase function, the chymase specific substrated (SUB) pro-Dala-Ang I contraction is completely by chymostatin.[75] Reproduced with permission of the American Heart Association.

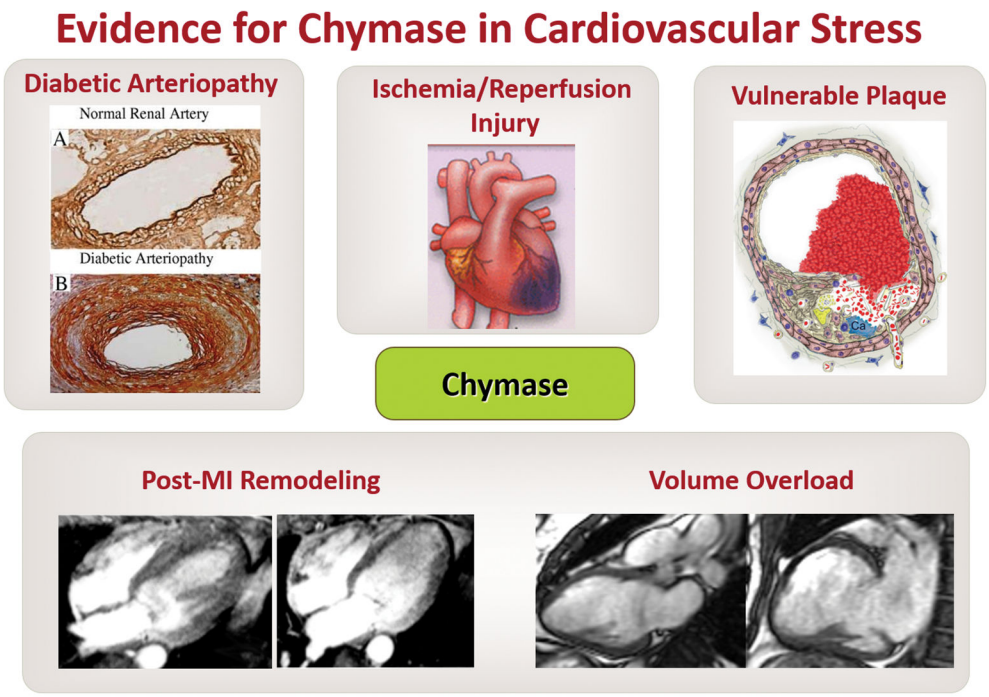

Figure 3. Evidence from human pathology and preclinical animal models supports the important role for chymase in cardiovascular stress

Upper left panel: demonstrates a cross-section of a human renal artery with marked increase in chymase (brown) extending from the endothelium to adventitia in a patient with diabetes (reproduced with permission of the American Heart Association).[252] Center and upper right panels: cartoons demonstrate the potential role of chymase in attenuating ischemia/reperfusion injury and promoting stablization of the vulnerable atherosclerotic plaque (reproduced with permission of the American Heart Association). Bottom right panel: demonstrate the marked increase in LV volume and LV wall thinning in a patient with chronic MR (right image) compared with a normal subject (left image) (reproduced with permission of the American Heart Association).[253] Bottom left panel: demonstrates the increase in LV volume and apical wall thinning in a patient 6 months after an antero-apical myocardial infarction (right image) compared with three days after myocardial infarction (MI; left image) (reproduced with permission of the American Heart Journal).[254] Targeting the multifunctional roles of chymase (Figure 1) in each of these conditions by chymase inhibition will complement conventional RAS blockade in promoting better outcomes and tissue protection.

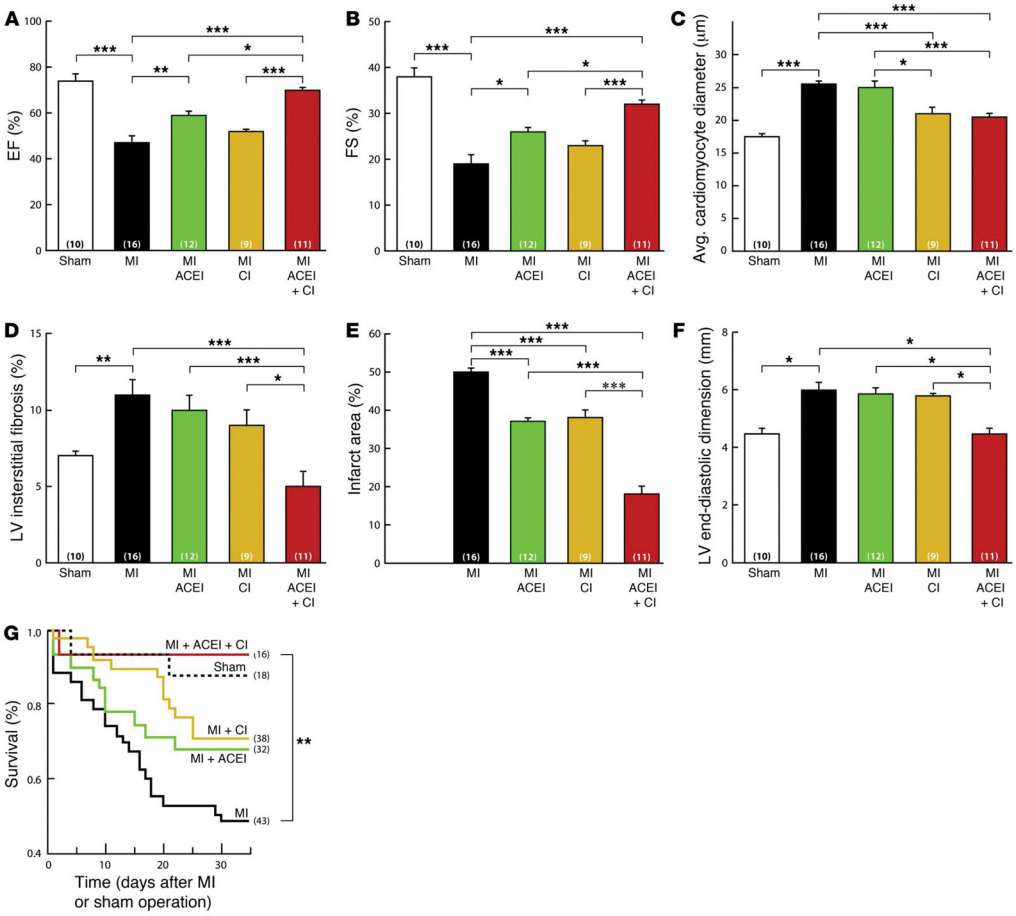

Figure 4. LV function and mortality after left anterior descending (LAD) artery occlusion in hamster

The combination of ACE inhibition (ACEi) and chymase inhibition (CI) produces the greatest improvement in LV ejection fraction (EF) (A), fractional shortening (FS) (B), cardiomyocyte diameter (C), LV fibrosis (D), infarct area (E), LV end-diastolic dimension (F), and survival (G) 1 month after LAD occlusion. Drug administration was started within 24 hours after LAD occlusion.[157] Reproduced with permission of the American Society of Clinical Investigation.

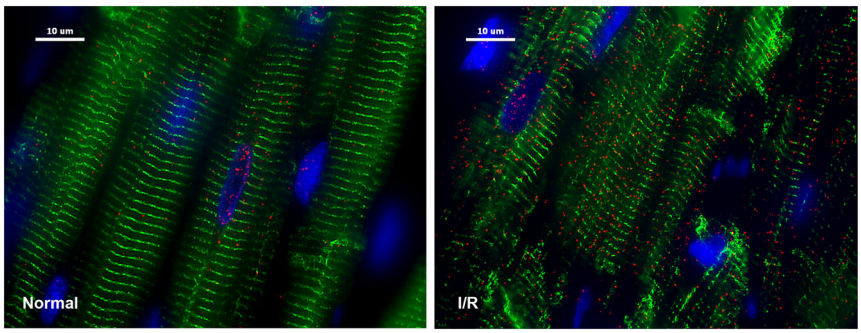

Figure 5. Chymase inside dog cardiomyocytes during ischemia/reperfusion

Adult dogs subjected to 60 minutes of LAD occlusion and 100 minutes of reperfusion (right panel) or normal controls (left panel). Ischemia/reperfusion LV led to a marked increase in chymase (red) in cardiomyocytes with breakdown of desmin (green, right). I/R: ischemia/reperfusion; blue: DAPI.[158] Reproduced with permission of Plos One.

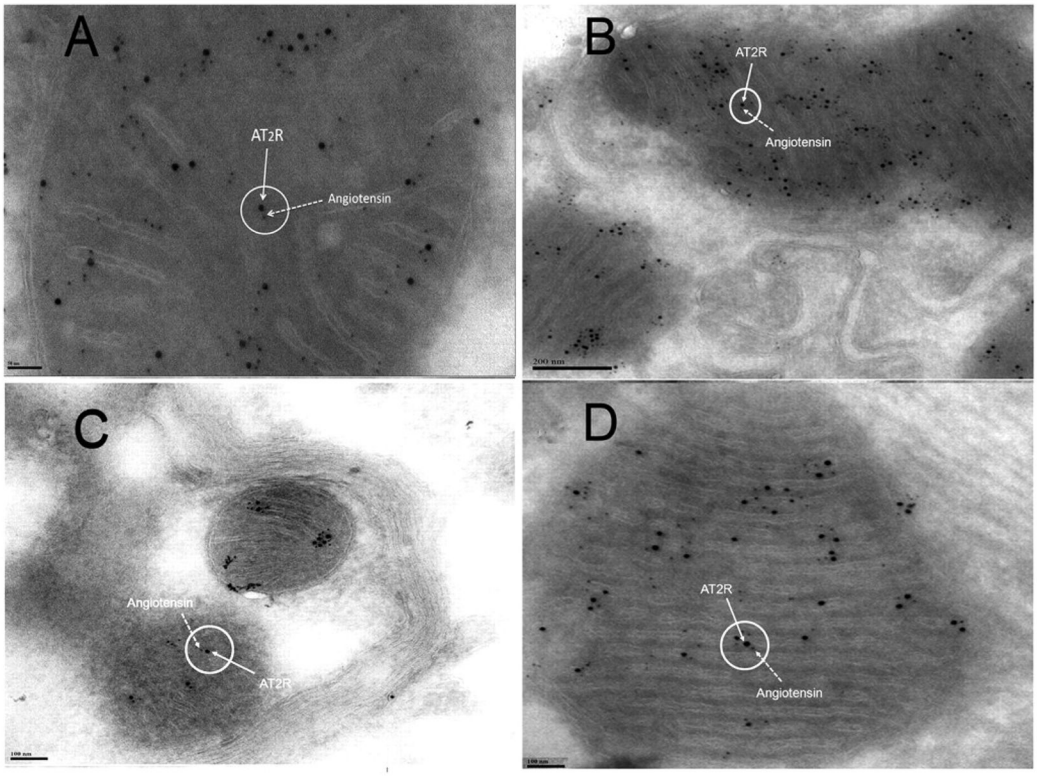

Figure 6.

Immunogold transmission electron microscopy using gold-labeled anti-AT_2R antibody (12 nm gold) and a gold-labeled anti-Ang antibody (6 nm gold) demonstrates the localization of the AT_2 receptor and binding thereof to Ang in the mitochondria of mouse hepatocytes (A), kidney tubular cells (B), neurons (C), and cardiac myocytes (D).[218] Reproduced with permission of *PNAS*.

Table 1

Chymase Upregulation in Human Disease.

Pathological Condition	Reference
Infarct coronary arteries	36–39
Atherosclerotic and aneurysmal aortas	40–49
Pathogenesis of angiogenesis in small cell lung cancer and tumor progression	51,52
Gastric cancer	53
Atrial myocardium of patients with ischemic and valvular heart disease	54
Idiopathic pulmonary fibrosis	55,56
Chronic obstructive pulmonary disease	57
Veins of arteriovenous fistula of patients and kidney with end stage renal disease	58,59
Hypertensive nephropathy	59
Kidney parenchyma of polycystic kidney disease	60
Coronary and renal arteries and kidney parenchyma of patients with diabetes	61–63
Rejected kidney allograft	65
Skin keloid	66,67
Preeclampsia	68–70
Submucosa and muscularis intestinal layers of patients with Crohn's disease	71
Atopic dermatitis	72

Table 2

Preclinical studies in mice, rats and hamsters

Condition	Model	Chymase Inhibition Effects	Reference
Intermittent Hypoxia	mouse	Decreased perivascular fibrosis and cardiomyocyte hypertrophy, inflammatory cytokines, oxidative stress, Ang II, and superoxide production in LV myocardium	104
Dextran sodium sulfate-induced colitis	mouse	Decreased neutrophil infiltration and MMP-9 activity resulting in improved disease activity index and histological scores	105
Ang II induced aneurysm	ApoE- deficient mouse	Decreased MMP-9 activity and macrophage infiltration. Prevented Ang II-induced aortic aneurysm formation in ApoE-deficient mice	106
Increased salt diet	mouse	Suppressed hypertension and decreased plasma Ang II and aldosterone	107
Myosin-immunized myocarditis	rat	Decreased MMP-9 activation, TGF-β expression, and macrophage infiltration; Improved survival and LV function 4 weeks after immunization	108
Stroke-Prone SHR Rat	rat	Decreased aortic MMP-9 activity, TNF-α, MCP-1 levels and macrophage infiltration; Improved vascular function *in vitro* and survival	111
Indomethacin- induced colitis	rat	Decreased intestinal wall MMP-9 activation and myeloperoxidase activity; Decreased intestinal lesions and damage	110
Lipopolysaccharide induced liver injury	hamster	Improved liver function and reduced liver necrosis and fibrosis; Decreased liver MMP-9 activation and myeloperoxidase and TNF-α levels	116
Carbon tetrachloride- induced chronic liver failure	hamster	Decreased liver myofibroblasts and fibrosis and decreased liver Ang II levels	117
Elastase-induced aneurysm formation	hamster	Decreased abdominal aortic aneurysm size and mast cell infiltration	118
Streptozotocin- induced diabetes	hamster	Decreased LV NOX4-induced oxidative stress, malonaldehyde levels, and interstitial fibrosis; Attenuated kidney oxidative stress, decreased renal fibrosis and TGF-β, and improved renal function	119–121
Cigarette smoking and bleomycin-induced lung injury	hamster	Decreased lung TGF-β signaling, ET-1 levels, and pulmonary hypertension and fibrosis	122,123
Coronary artery ligation	hamster	Improved LV systolic function and hemodynamics, hypertrophy and fibrosis; Improved survival	124–126
Obstructed kidney	hamster	Attenuated tubulointerstitial fibrosis and TGF-β and α-smooth muscle actin expression	127
Prolonged high-fat diet	hamster	Prevented lipid deposition in the aortas	128