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Mast Cell Chymase and Tryptase as Targets for Cardiovascular and Metabolic Diseases

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Abstract

Mast cells are critical effectors in inflammatory diseases, including cardiovascular and metabolic diseases and their associated complications. These cells exert their physiological and pathological activities by releasing granules containing histamine, cytokines, chemokines, and proteases, including mast cell-specific chymases and tryptases. Several recent human and animal studies have shown direct or indirect participation of mast cell-specific proteases in atherosclerosis, abdominal aortic aneurysms, obesity, diabetes, and their complications. Animal studies have demonstrated the beneficial effects of highly selective and potent chymase and tryptase inhibitors in several experimental cardiovascular and metabolic diseases. In this review, we summarize recent discoveries from *in vitro* cell-based studies to experimental animal disease models, from protease knockout mice to treatments with recently developed selective and potent protease inhibitors, and from patients with preclinical disorders to those affected by complications. We hypothesize that inhibition of chymases and tryptases would benefit patients suffering from cardiovascular and metabolic diseases.

Keywords

Mast cell; chymase; tryptase; atherosclerosis; abdominal aortic aneurysms; obesity; diabetes

INTRODUCTION

Mast cells, like other inflammatory cells such as macrophages and T cells, are essential in mediating the inflammatory process. When activated, mast cells rapidly release characteristic granules and various hormonal mediators into the interstitium. These mediators include histamine, proteoglycans, cytokines (e.g., interleukin-6 [IL-6], tumor necrosis factor-α [TNF-α], and interferon-γ [IFN-γ]), mast cell-specific proteases (tryptase, chymase, and carboxypeptidase A), and other proteases (cysteinyl cathepsins and matrix metalloproteinases [MMPs]) [1, 2] that participate in the pathogenesis of atherosclerosis, abdominal aortic aneurysms (AAAs), obesity, and diabetes [3–15]. As mast cell-specific proteases, both chymase and tryptase belong to the serine protease family, while carboxypeptidase A is a zinc-dependent metalloproteinase. Their three-dimensional

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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structures, substrate cleavage specificity, common substrates, and *in vivo* functions are well reviewed elsewhere [16]. Chymase can mediate the conversion from angiotensin-I (Ang-I) into angiotensin-II (Ang-II) [17–19]; generate fibronectin and transforming growth factor-β (TGF-β) from extracellular matrix (ECM) [20, 21]; process collagen for fibril formation from type I procollagen [22]; activate MMPs from their zymogens [23, 24]; and activate IL-1β, IL-18, endothelin-1, and endothelin-2 from their latent forms [25–27]. Chymase also degrades lipoproteins, thereby promoting macrophage foam cell formation [28]. Tryptase activates pro-MMPs [29] and degrades chemokines [30], lipoproteins [31], and fibronectin [32]. Mast cell-derived chymase and tryptase also are implicated in collagen synthesis and tissue fibrosis [33–35], angiogenesis [36, 37], and immunoglobulin molecule synthesis [38] — all of which associate closely with the pathophysiology of cardiovascular and metabolic disorders [39–44].

In this review, we briefly summarize our current understanding of the functions of mast cell chymases and tryptases in cardiovascular and metabolic diseases, mainly in atherosclerosis, AAA, obesity, and diabetes, including protease functions and drug developments against these proteases.

CHYMASES AND TRYPTASES IN CARDIOVASCULAR DISEASES

Mast cells' participation in cardiovascular diseases was first implicated more than half a century ago [45–47]. Great progress has been made over the past decades, and much of our basic knowledge regarding these cells has come from studies led by Dr. Petri Kovanen and his colleague Dr. Ken Lindstedt. Their work provided a basic understanding of how mast cells, as a whole or as individual components, may affect different arterial cells or lipid proteins, thereby contributing to atherogenesis. For example, mast cells release heprin proteoglycan to bind to apolipoprotein B (apoB) from low-density lipoprotein (LDL) or release neutral proteases to degrade apoB, thereby facilitating LDL accumulation in macrophages and finally, foam cell formation [48, 49]. The same heprin proteoglycan also mediates LDL accumulation in smooth-muscle cells (SMCs) and promotes SMC foam cell formation [50] or inhibits SMC proliferation [51], which contribute importantly to media SMC loss and arterial wall thinning. Mast cells are also rich sources of growth factors. By releasing pro-angiogenic factor basic fibroblast growth factor (bFGF), mast cells may contribute to neovascularization. Indeed, in human atherosclerotic lesions, bFGF-positive mast cells are localized to macrovesssels in both intima and adventitia [52]. Direct participation of mast cells in cardiovascular diseases, however, has only recently been established using experimental animals. Using mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice and atherosclerosis-prone low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mice on a C57BL/6 background, and aortic elastase perfusion-induced experimental AAA, we demonstrated that mast cells participate in both atherosclerosis and AAA by releasing proinflammatory cytokines, chemokines, and proteases to induce inflammatory cell recruitment, arterial cell apoptosis, angiogenesis, and matrix protein remodeling [53, 54]. Pharmacological stabilization of mast cells with their inhibitor, cromolyn (disodium cromoglycate, DSCG), reduced mast cell activation-induced atherosclerotic plaque intraplaque hemorrhage, macrophage apoptosis, vascular leakage, and CXCR2/VLA-4 (Very Late Antigen-4)-mediated recruitment of leukocytes in apolipoprotein E-deficient ($Apoe^{-/-}$) mice [55]. Mast cell stabilizers MY-1250 and cromolyn also can prevent mast cell-mediated macrophage foam cell formation *in vitro* [56]. We showed that mast cell stabilization with cromolyn reduced elastase perfusion-induced AAA [54], which suggests that mast cell-derived cytokines, growth factors, proteoglycans, chymases and tryptases, or other proteases — such as cysteinyl cathepsins and MMPs — participate directly and indirectly in the pathogenesis of atherosclerosis and AAA. Several recent articles summarize these findings [57–59].

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CHYMASES AND TRYPTASES IN ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease of the arterial wall, caused largely by the accumulation of macrophages, or foam cells, that are enriched with intracellular cholesterol and lipid promoted by low-density lipoprotein (LDL) without adequate removal of fats and cholesterol from the macrophages by functional high-density lipoprotein (HDL). In addition to macrophages, monocytes, neutrophils, lymphocytes, and mast cells also reside in the arterial intima, many of them in close proximity to foam cells [60] — suggesting that these inflammatory cells are involved in the transformation of macrophages into foam cells.

Ihara and colleagues found high levels of Ang-II forming activity and chymase expression in human atherosclerotic lesions [47]. Several pieces of pioneering work demonstrated important effects of mast cell chymase and tryptase on arterial wall macrophages, SMCs, and endothelial cells (ECs). By degrading apoE or HDL3 components, such as apolipoprotein AI (apoAI), apoA2, preβ1LpA1, and LpA4, mast cell chymase abolishes HDL3 activities in cholesterol efflux from macrophage foam cells [60–63]. Mast cell tryptase also degrades HDL3 and impairs cholesterol reverse transport, a process facilitated by proteoglycan [31]. By degrading SMC matrix protein fibronectin and disrupting SMC focal adhesion [64] and by disrupting the NF-κB-mediated survival-signaling pathway [65], mast cell chymase induces SMC apoptosis [66], providing a mechanistic explanation of chymase contribution to aortic wall media SMC loss and thinning during atherogenesis. In addition, chymase inhibits SMC growth and collagen synthesis [67]. Mast cells release chymase (and carboxypeptidase A) and degrade endothelin-1 from ECs [68], and therefore affect normal vasodilation. Both chymase and TNF-α contribute to EC apoptosis. While TNF-α triggers EC apoptosis by translocating cytochrome C from mitochondria into cytoplasm [69], chymase induces EC apoptosis by degrading EC matrix prote*in vitro*nectin and fibronectin and inactivating focal adhesion kinase (FAK)-mediated cell survival signaling [70]. Therefore, mast cell proteases affect the pathobiology of macrophages, SMCs, and ECs. To demonstrate a direct role of mast cell proteases in atherosclerosis, we used bone marrow-derived mast cells (BMMCs) from chymase-deficient [71] and tryptase-deficient [72] mice, and proved that both proteases promote aortic SMC apoptosis. While BMMCs from wild-type (WT) mice induced mouse aortic SMC apoptosis, those from chymase-deficient or tryptase-deficient mice showed no activity in inducing SMC death. Although not tested in our studies due to technical difficulties, BMMCs from chymase-deficient or tryptase-deficient mice may also have impaired activities in inducing EC apoptosis. Therefore, mast cell proteases may contribute to plaque erosion and complications of atherosclerosis by inducing vascular cell apoptosis. *In vitro*, treatment of human coronary arteries intraluminally with recombinant tryptase or chymase induced endothelial damage, as characterized by disruption of EC adhesion followed by retraction and desquamation [73].

Chymase- and tryptase-mediated bioactivation of pro-enzyme and latent cytokines also appears important to atherogenesis. While chymase mediates pro-MMP-9 activation [24, 74], tryptase activates MMP-1, −2, and −3 [75–77]. All of these metalloproteinases have been implicated in promoting the pathogenesis of atherosclerosis and AAA [78–82]. Chymase also activates latent TGF-β1 from mast cells themselves, and those exogenously added to the mast cell culture [83] or from extracellular matrix of cultured epithelial cells or ECs [21]. Active TGF-β1 may then cause endothelial dysfunction via stimulation of reactive oxygen species (ROS) production by the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. Transgenic overexpression of TGF-β1 accelerates atherosclerosis and hypertension in *Apoe*$^{-/-}$ mice [84], and adenovirus-mediated overexpression of TGF-β1 induces formation of cellular and matrix-rich intima in mouse carotid arteries, due to enhanced SMC migration and matrix deposition [85].

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Several studies, including our own [86], have established an association of blood tryptase levels with atherosclerotic plaque instability [87, 88]. Patients with acute myocardial infarction (MI) or unstable angina pectoris have significantly higher serum tryptase levels than those without significant coronary heart disease or with stable angina pectoris. Serum chymase levels were also higher in patients with acute MI or unstable angina pectoris than in patients with stable angina pectoris or those without significant coronary heart disease [86]. In mice, both chymases and tryptases control vascular wall atherosclerosis-pertinent cathepsin expression [71, 72]. Monocytes from mouse tryptase mMCP (mouse mast cell protease)-6-deficient mice [72] and SMCs from mouse chymase mMCP-4-deficient mice [71] have significantly reduced expression and activities of cathepsins B, S, K, and L. Mast cell chymases and tryptases therefore may directly and indirectly participate in atherogenesis. Some selected activities of chymase and tryptase in atherogenesis are summarized in (Fig. **1**).

CHYMASES AND TRYPTASES IN AAA

The arterial wall histopathological changes of AAA include accumulation of lipids in foam cells, extracellular free cholesterol crystals, calcifications, thrombosis, angiogenesis, adventitial inflammatory cell infiltration, and ulcerations and ruptures of the endothelium layers. Inflammatory cells — including macrophages, lymphocytes, neutrophils, and mast cells — produce cytokines and proteases to promote inflammatory reactions, aortic medial SMC apoptosis, extracellular matrix degradation, and neovascularization [89], all of which associate closely with AAA pathogenesis.

We found that mast cells induce vascular cell — SMC and EC — protease expression by releasing their inflammatory cytokines (IL-6, TNF-α, and IFN-γ), thereby enhancing angiogenesis, vascular cell apoptosis, and ECM degradation [54]. Mäyränpää and colleagues investigated the relationship between mast cells and inflammation, neovascularization, and intraluminal thrombus in human AAA, and their results support the direct participation of mast cells in the pathogenesis of AAA — particularly regarding neovascularization of the aortic wall [90].

We demonstrated significant correlations of serum chymase [71] and tryptase levels with AAA annual expansion rate [72]. More interesting discoveries include that high serum tryptase levels significantly increased the risks of later surgical repair and overall mortality in a patient follow-up study [69]. Both mast cell proteases were expressed highly in the media and adventitia of human AAA, but were negligible in normal aortas, as determined by immunohistology and immunoblot analysis. In an elastase perfusion-induced mouse AAA model, mice lacking connective tissue chymase mMCP-4 [71] or tryptase mMCP-6 [72] developed significantly smaller AAA lesions than did WT control mice. Mechanistically, we found that chymase contributed to microvessel growth, vascular SMC apoptosis, and vascular cell cysteinyl cathepsin expression and activities. Compared with mast cells from WT mice, those from chymase mMCP-4-deficient mice had significantly impaired induction of microvessel sprouting in an aortic ring assay, aortic SMC apoptosis, and cathepsin expression and activities in ECs and SMCs [71]. In contrast, tryptase contributed to SMC apoptosis, vascular EC and SMC cathepsin expression, and monocyte transmigration, but showed no effect on microvessel growth [72]. These animal experiments supported a direct participation of mast cell chymase and tryptase in AAA formation and progression. But both chymase and tryptase may also contribute indirectly to AAA pathogenesis. As discussed above, chymase-deficient SMCs have reduced expression and activities of cathepsins B, L, K, and S [71]. Monocytes from tryptase-deficient mice also demonstrate reduced expression of the same set of cathepsins, as determined by real-time polymerase chain reaction [72]. Mast cells from tryptase-deficient mice show reduced activities of cathepsins B, K, L, and S,

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as determined by cathepsin active site labeling assay [72]. Our recent studies using aortic elastase perfusion-induced experimental AAA or Ang-II infusion-induced experimental AAA proved important roles of cathepsins K [4], L [3], and S [91] in AAA pathogenesis. Absence of any one of these proteases significantly suppressed AAA formation and progression. Therefore, reduced AAA in chymase- or tryptase-deficient mice [71, 72] may be due partially to reduced expression or activities of these AAA-pertinent cathepsins. Although not published, we detected great reduction of intracellular cytokine/chemokine levels in mast cells from chymase-deficient mice. Therefore, reduced activities of chymase-deficient mast cells in inducing neovascularization and SMC apoptosis could also be partially due to impaired production of pro-inflammatory cytokines from these mast cells — a hypothesis that merits further investigation.

Conversely, mast cell chymase and tryptase activities may also explain AAA formation studied in other classes of proteases or inflammatory molecules. For example, cathepsin C participates in AAA formation by activating neutrophil proteases, and thereby controlling neutrophil chemokine CXCL2 production and neutrophil recruitment [10]. Although this study did not test whether mast cells or mast cell proteases participate in cathepsin C-mediated AAA formation, we know that cathepsin C activates chymase from its pro-enzyme in mast cell granules [92], thereby enhancing mast cell Ang-II production, which ultimately induces vascular wall expression of MMP-9, another well-known protease involved in AAA pathogenesis [82]. Reduced AAA formation in cathepsin C-deficient mice therefore also may result from reduced activities of MMP-9 and chymases.

MAST CELL CHYMASES AND TRYPTASES IN METABOLIC DISEASES

Although direct participation of chymases or tryptases in obesity or diabetes has not been tested in any experimental models, cysteinyl protease cathepsins — which are indirectly regulated by chymase and tryptase [71, 72] — have been confirmed in diet-induced and genetically generated experimental obesity and diabetes. Mice deficient in cathepsins L or K, or WT mice treated with cathepsin L- or K-selective small molecule inhibitors, are leaner than control mice or have significantly improved glucose sensitivity [5, 6]. Our recent studies demonstrated that mast cells participate directly in obesity and diabetes [93]. Very few studies have studied mast cells in obesity. White adipose tissue (WAT) contains high numbers of tryptase-positive mast cells [93] or mast cell progenitor cells [7]. Mast cell-derived prostaglandins (PGs) metabolite 15-deoxy-delta-12,14-PGJ(2) (15-deoxy-delta PGJ(2)) is essential to mast cell-induced adipogenesis of 3T3-L1 cells [94]. Although not reported, we found that mast cells from chymase mMCP4-deficient mice had impaired induction of 3T3-L1 cell differentiation (Shi, unpublished observation). Chymase and tryptase therefore may participate directly or indirectly in the development of obesity. In contrast, mast cell functions in diabetes mellitus have been much more thoroughly investigated. Placentae from pregnant women who had class C diabetes (diabetes developed between 10–19 years of age without vascular complications) contain significantly higher numbers of mast cells and associated vascular endothelial growth factor (VEGF) expression than do those from gestationally matched controls [95]. As we will discuss further, mice lacking mast cells or receiving mast cell stabilizers demonstrated enhanced glucose and insulin sensitivities in diet-induced obese mice. In non-obese diabetic (NOD) mice, antibody against Fcε receptor-1, the high affinity receptor of immunoglobulin E (IgE), delayed type 1 diabetes onset [96].

Vascular hypertrophy is a feature of experimental and human diabetes. Streptozotocin-induced diabetic rats showed significant increases in mesenteric artery weight, wall-to-lumen ratio, arterial wall ECM deposition, and gene expression of epidermal growth factor (EGF) and TGF-β1 [97]. In those rats, diabetes also associated with an increase of mast cell

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numbers to the tongues [98] and in mesenteric vessels [97] — often associated with fibrosis, a common feature of diabetic microvascular complications [35]. In those experimental diabetic rats, treatment with the mast cell degranulation inhibitor tranilast (N-[3,4-demethoxycinnamoyl]-anthranilic acid) reduced mesenteric vessel fibrosis, artery weight, wall-to-lumen ratio, and matrix deposition, although it did not influence tryptase-positive mast cell infiltration, plasma glucose level, or systolic blood pressure [35, 99]. *In vitro*, tranilast greatly suppressed glucose-induced insulin secretion from cultured rat pancreatic β-cell line INS-1E cells and rat islets, and enhanced INS-1E cell glucose uptake [100]. Conversely, insulin and insulin-like growth factor-1 (IGF-1) promote mast cell survival in the absence of IL-3 in a PI3K–dependent manner [101], and high glucose increases mast cell intracellular ROS levels and expression of β-hexosaminidase and proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-13) [102] — among which, IL-6 is a well-known inducer of mast cell chymases and tryptases [103–105]. These observations support strongly an essential role of mast cells, and possibly of their proteases, in obesity and diabetes.

CHYMASES AND TRYPTASES IN OBESITY

Like atherosclerosis and AAA, obesity is an inflammatory disease. In addition to adipocytes, inflammatory cells — including macrophages, T cells, neutrophils, and mast cells — play essential roles in the pathogenesis of obesity.

WAT may represent an important source of mast cells in physiological and pathological situations [7]. Staining human WAT sections with a mast cell tryptase monoclonal antibody revealed high numbers of mast cells in WAT from obese subjects, compared with that of lean subjects. Higher numbers of mast cells were also found in epididymal fat tissue from obese mice compared with that of lean subjects, by staining with a monoclonal antibody against mouse mast cell protease-6 (mMCP6) [106], a human tryptase homologue. In our study of 80 obese patients and 32 lean controls, we found that mast cell-specific serum tryptase concentration was significantly higher in obese subjects than in lean individuals ($P = 0.001$) [93]. In a more recent study of a general population of 1,216 people, Fenger *et al.* demonstrated that serum tryptase levels associated strongly with age ($P < 0.0001$, $P < 0.0001$), male sex ($P = 0.0012$, $P = 0.012$), and body-mass index (BMI) ($P < 0.0001$, $P = 0.037$) before and after adjustment for age, sex, BMI, serum HDL, alcohol consumption, smoking, and atopy status [107]. While subjects with BMI < 25 had serum tryptase levels at 3.3 μg/L, those with BMI > 30 had serum tryptase levels at 4.4 μg/L, $P < 0.0001$ [107]. These observations suggest a possible association between mast cells or mast cell proteases with obesity [93, 107].

Direct participation of mast cells in obesity was established using mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. These mice, after consuming a Western diet for 12 weeks, gained significantly less body weight and had improved glucose intolerance and reduced adipose tissue inflammation, with reduced leptin and insulin levels in the circulation, compared with congenic WT controls. Consistently, WT mice receiving a daily intraperitoneal injection of the mast cell stabilizer DSCG also had attenuated body weight gain. Adoptive transfer experiments of different cytokine deficient mast cells into $Kit^{W\text{-}sh/W\text{-}sh}$ mice demonstrated that mast cells contributed to diet-induced obesity by producing the inflammatory cytokines IL-6 and IFN-γ. $Kit^{W\text{-}sh/W\text{-}sh}$ mice receiving BMMCs from $Il6^{-/-}$ mice and $Ifn\gamma$ mice, but not WT mice or $Tnf^{-/-}$ mice, had significantly reduced body weight gain and improved glucose tolerance. In addition to releasing cytokines, mast cells may contribute to obesity by promoting angiogenesis. Mast cells are often localized next to the microvessels in WAT. The numbers of microvessels correlated positively with the increases in mast cell numbers during the development of obesity. WAT and muscle tissues from WT obese mice showed substantial immunostaining of CD31 (microvessels) and KIT (mast cells) — significantly

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more than those from WT lean mice [93]. Although not tested, high numbers of mast cells in WAT from obese mice may result from enhanced mast cell proliferation, reduced mast cell apoptosis, or increased mast cell recruitment — all of which may contribute to angiogenesis in WAT.

Indeed, we detected significantly higher levels of monocyte chemoattractant protein-1 (MCP-1) in WAT from obese mice than in WAT from lean mice [93]. $Kit^{W\text{-}sh/W\text{-}sh}$ mice fed a Western diet, or those receiving DSCG, had smaller CD31-positive areas, similar to those from chow diet-fed lean mice, compared with those in WT control mice not receiving medication. Reduced angiogenesis in $Kit^{W\text{-}sh/W\text{-}sh}$ mice or those receiving DSCG resulted in high numbers of apoptotic cells in WAT and muscle tissues. Mast cells contain cell type-specific chymases and tryptases. Although no data suggest that these mast cell proteases participate directly in obesity, the findings in our recent AAA study indicate that mast cell chymase plays an important role in angiogenesis. While BMMCs from WT mice promoted microvessel sprouting in an aortic ring assay, those from chymase-deficient mice showed significantly reduced potency to microvessel sprouting [71]. Besides mast cell-specific chymases and tryptases, mast cells are important reservoirs of MMPs and cysteine proteases, such as cathepsins S, K, and L [2] — all of which can regulate neovascularization, cell survival [108], and ECM remodeling essential for WAT growth. Both chymase and tryptase may also regulate the expression of these cysteinyl cathepsins in WAT [71, 72] (Fig. **1**). Indeed, WAT protein extracted from obese WT mice contained higher activities of cathepsins B, S, and L than that from $Kit^{W\text{-}sh/W\text{-}sh}$ mice [93].

MAST CELL CHYMASES AND TRYPTASES IN TYPE 1 DIABETES

Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia and associated with microvascular and macrovascular complications. Type 1 and type 2 are the most common types of diabetes. Although mast cells have been implicated in diabetes and its associated complications, few studies have focused on mast cell proteases. In a streptozotocin (STZ)-induced type 1 diabetes in hamsters, Maeda and colleagues [109] found that STZ induced renal chymase expression, accompanied by increased intrarenal Ang-II levels, overexpression of TGF-β and fibronectin in glomeruli, and renal mesangial expansion and deteriorated proteinuria. A selective chymase inhibitor, TEI-F00806, completely ameliorated the pathological changes of diabetic nephropathy, independent of blood pressure levels. The ACE inhibitor ramipril, which also inhibits Ang-II formation, did not show such therapeutic effects, supporting the participation of chymase in SZT-induced diabetic nephropathy. Shinji and colleagues [110] used the same experimental type 1 diabetes model and showed increased blood glucose levels and pancreatic chymase and total Ang-II-forming activities. Chymase inhibition with TY-51469 significantly reduced blood glucose levels and pancreatic chymase and total Ang-II-forming activities, along with significantly more pancreatic islets in the TY-51469 group than in the placebo group. Chymase activity therefore contributes to STZ-induced pancreatic islet disorganization.

MAST CELL CHYMASES AND TRYPTASES IN TYPE 2 DIABETES

We have shown that mast cells participate importantly in the pathogenesis of diet-induced type 2 diabetes. Glucose and insulin tolerance assays confirmed that deficiency or inhibition of mast cells improved these parameters, whereas diabetic mice had high serum insulin and glucose levels and high KIT positive mast cells in WAT [93]. In our recent Pre-diabetes Intervention Project involving 80 patients with confirmed diabetes, 189 patients with pre-diabetes, and 71 normal controls selected from 3163 volunteers, we found that both chymase and IgE levels in the blood were significantly higher in diabetic patients, followed by those with pre-diabetes, than those from controls with normal blood glucose levels. In an ordinal

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logistic model, interactions between IgE and chymase greatly increased the risk (odds ratio, OR) of developing diabetes before (OR: 2.479 [1.079–5.778], $P = 0.033$) and after (OR: 2.594 [1.118–6.018], $P = 0.026$) adjustment for age, sex, hypertension, waist circumference, waist-to-hip ratio, BMI, total cholesterol, triglyceride, HDL, LDL, hyperinsulinemia, and homeostatic model assessment indexes [111]. Although not statistically significant, interactions between IgE and tryptase also increased the risk of developing diabetes by 2.091 ($P = 0.068$) and 2.167 ($P = 0.057$) folds, before and after the same adjustment [111]. Among the same population, when patients with pre-diabetes — including isolated impaired fasting glucose (I-IFG), isolated impaired glucose tolerance (I-IGT), and mixed IFG/IGT — were considered, we found that plasma chymase levels associated with an increased risk of I-IGT (OR: 2.862 [1.186–6.907], $P = 0.019$) and mixed IFG/IGT (OR: 3.142 [1.310–7.541], $P = 0.010$). After adjustment for age, sex, and BMI, high plasma chymase levels remained significantly associated with an increased risk of I-IGT (OR: 3.057 [1.231– 7.590], $P = 0.016$) and mixed IFG/IGT (OR: 2.127 [1.218–8.030], $P = 0.018$) (Shi, unpublished data). These data suggest that mast cells and mast cell chymase and tryptase are potential drug targets for human diabetes or pre-diabetes — a hypothesis currently being tested in human clinical trial.

MAST CELL CHYMASES AND TRYPTASES IN DIABETIC COMPLICATIONS

Mast cells, which localize to various organs including the lungs, heart, and kidneys [112–114], also play a central role in the pathogenesis of diabetic complications. Mast cells infiltrate the kidney and got degranulated in renal diseases [115]. The degranulation of mast cells releases pathological substances like TGF-β, chymase, tryptase, cathepsin G, histamine, renin, and various inflammatory cytokines [116–119], which may play a detrimental role in the pathogenesis of diabetic nephropathy. The level of chymase increases in diabetic nephropathy, and this increase associates with glomerulosclerosis, tubulointerstitial fibrosis, and vascular fibrosis in patients with diabetic nephropathy [120, 121]. Chymase converts Ang-I into Ang-II, which may play a critical role in diabetic vascular disease [122]. Increased chymase associates with the accumulation of advanced glycation end products (AGEs) in diabetic renal vasculature [122]. Moreover, chymase participates in the conversion of pro-MMP-9 to MMP-9 [123], a protease implicated in diabetic nephropathy and diabetic retinopathy [124, 125]. All these activities suggest that mast cell chymase participates in the pathogenesis of diabetic complication [126], although direct evidence is currently not available.

Tryptase enhances the production of VEGF [127], which is implicated in the development of diabetic nephropathy [128]. Notably, mast cells are a major source of tryptase [129], and renal mast cell degranulation-mediated release of tryptase may play a detrimental role in the pathogenesis of diabetic nephropathy. This contention is supported by the participation of mast cell tryptase in the development of renal interstitial fibrosis, by increasing the production of ECM proteins [130].

CHYMASE-GENERATED ANG-II IN CARDIOVASCULAR DISEASES

Ang-II, an end product of the renin-angiotensin system (RAS), exerts a wide range of physiological and pathological effects on the cardiovascular, renal, endocrine, and nervous systems. Ang-II regulates blood pressure through its action on multiple target receptors, mainly Ang-II receptor type 1 (AT_1) and type 2 (AT_2) on the vascular wall, kidney, and adrenal gland. Octapeptide Ang-II is converted from Ang I through the cleavage of the Phe^8–His^9 bond by angiotensin-converting enzyme (ACE) both within the circulatory system and within tissues. But chymase is an important alternative pathway of Ang-II conversion within the cardiovascular tissues [131]. The activities of Ang-II from the

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chymase-depend pathway are almost the same as those from the ACE-dependent pathway, except that Ang-II from the chymase-dependent pathway may not regulate blood pressure. Unlike ACE, chymase may generate and degrade Ang-II, depending on the species. For example, human chymase generates Ang-II without further degradation, where as chymase from other species — including dog, hamster, rat, and mouse — also generates Ang-II, but degradation follows in a time-dependent manner [132].

Ang-II from the chymase-dependent pathway has been implicated in vascular proliferation and the pathogenesis of aortic valve diseases, MI, heart failure, and AAA. In a dog model of percutaneous coronary intervention, chymase activity — but not that of ACE — increased after balloon catheter injury [44]. In this model, angiotensin-receptor blockers (ARB; candesartan) and chymase inhibitor (NK3201), but not ACE inhibitor (enalapril), prevented vascular proliferation [44, 131]. In experimental coronary artery bypass grafting, Ang-II formation in the grafted veins seemed to depend mainly on chymase activity. Chymase-produced Ang-II induced post-grafting expression of fibronectin, and collagen types I and III. Chymase inhibitor Suc-Val-Pro-Phe$^{P}(OPh)_2$ greatly suppressed these matrix protein expressions and post-grafting vascular proliferation [133]. Chymase-generated Ang-II may also exert proinflammatory and profibrotic effects. With its activities in inducing vascular cell MCP-1 expression [134, 135] and in inducing TGF-β expression and consequent collagen synthesis [136], Ang-II may potentially contribute to the pathogenesis of calcific aortic valve disease. Indeed, stenotic aortic valves from patients undergoing valve replacement surgery contain greatly increased mRNA and protein levels of AT1 receptor and chymase [137].

In tachycardia-induced heart failure in dogs, the left ventricle (LV) showed increased chymase-positive mast cells. Chymase inhibitor SUNC8257 reduced LV mast cell numbers and cardiac Ang-II levels, suppressed LV expression of TGF-β and collagen types I and III, and decreased LV fibrosis and LV end-diastolic pressure [138]. Similar observations were obtained in experimental MI animals. Chymase-activated TGF-β is a major stimulator of myocardial fibrosis. In cultured fibroblasts, addition of chymase increased fibroblast proliferation and media TGF-β levels, all of which can be suppressed by a chymase inhibitor [139]. Cardiac tissue chymase and associated Ang-II levels are increased in hamsters 1 day after MI [140]. Chymase inhibitor [141, 142] and ARB [140] both attenuated cardiac dysfunction and extended survival.

In elastase infusion-induced AAA in hamsters and mice, chymase and Ang-II-forming activities were increased [41, 143], and were reduced by the chymase inhibitor NK3201 [143]. In *Apoe*$^{-/-}$ mice, either Ang-II or chymase infusion induced AAA. Ang-II-induced AAA had increased expression of chymase and MMP-9, and their activities were inhibited by NK3201 [144].

CHYMASE-GENERATED ANG-II IN METABOLIC DISEASES

Ang-II activities in obesity have been throughly studied [145]. The expression of renin, ACE, and AT_1 genes significantly increased in adipocytes from obese hypertensive patients [146] and WAT from obese patients contain increased numbers of mast cells [93]. These observations suggest a role of chymase in Ang-II production in WAT. How important chymase-generated Ang-II is in WAT as compared with that from the ACE pathway, however, remains unknown. In contrast, several studients have implicated chymase-generated Ang-II directly and indirectly in diabetes in several studies.

In cultured vascular SMCs, high glucose and AGEs — a key molecule accumulated in the microvasculature in the development of diabetic retinopathy [147, 148] — induced

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chymase-dependent, but not ACE-dependent, Ang-II formation via ERK1/2 MAP kinase activation [149], suggesting that in vascular tissues from diabetic patients, high glucose and AGEs may induce chymase-dependent Ang-II formation. Ang-II promotes retina VEGF expression [26]. In diabetic patients, VEGF is important in the initiation and development of diabetic retinopathy, by regulating vascular permeability, angiogenesis, and EC proliferation. The vitreous of eyes from patients with diabetic retinopathy contains increased levels of ACE, VEGF, and MMP9 [150]. ABR treatment inhibited AGE and VEGF expression in diabetic rats [151], indirectly suggesting that chymase inhibition also prevents diabetic retinopathy. In STZ-induced type 1 diabetes in hamsters, chymase and Ang-II–forming activities are increased in the pancreas [110]. After STZ injection, pancreatic islets were reduced. Chymase inhibitor TY-51469 reduced glucose levels, inhibited the chymase and total Ang-II–forming activities, suppressed oxidative stress, and maintained pancreatic islet numbers [110].

In type 2 diabetic animals [152] and human patients with evidence of vascular diseases or diabetes [153, 154], Ang-II contributes to islet disorganization and high risk for cardiovascular events. ACE inhibitors or AT1 receptor antagonists improved islet fibronogenesis, apoptosis, and oxidative stress; diabetes-associated complications; and cardiovascular morbidity and death. Chymase inhibitors, therefore, may be useful in preventing, or lowering the risk for, type 2 diabetes [155].

CHYMASE AND TRYPTASE INHIBITORS IN CARDIOVASCULAR DISEASES

Although both chymases and tryptases are essential to mast cell biology and to the pathogenesis of atherosclerosis, AAA, and associated complications, most current studies focus on the development of chymase inhibitors (Table **1**) in experimental cardiovascular diseases in animals. Tryptase inhibitors receive much less attention, but this does not mean that tryptase is less important than chymase. We demonstrated that mouse tryptase mMCP-6-null mice are resistant to elastase perfusion-induced AAA [72]. In THP-1-derived macrophages and primary human monocyte-derived macrophages, the tryptase inhibitor APC-366 blocked ox-LDL-induced foam cell formation. Mechanistically, APC-366 inhibited tryptase activities can block reduction of nuclear receptor LXRa (regulates lipid homeostasis) expression, can increase the expression of ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1; involved in the cholesterol efflux pathway and macrophage foam cell formation), and can increase sterol regulatory element binding protein-1c (SREBP-1c) (regulates gene for de novo lipogenesis) expression [156].

CHYMASE INHIBITORS IN ATHEROSCLEROSIS

As summarized in (Table **1**), at least three chymase inhibitors have been tested in atherosclerosis and related conditions. In a dog vein graft disease experimental model [157], each animal underwent right common carotid artery bypass grafting with the ipsilateral external jugular vein. Grafting increased vascular cell proliferation, Ang-II forming activity, chymase activity, ACE activity, and intimal thickening (intima/media ratio) 28 days after operation. When dogs received chymase inhibitor NK3201 orally, however, all such phenotypes were significantly suppressed. The same group performed a similar experiment using a balloon catheter-induced carotid artery injury model in dogs to test the role of NK3201 in intimal hyperplasia [44]. Carotid injury significantly increased artery chymase activity. NK3210 suppressed artery chymase activity, but had no effect on artery ACE activity, plasma renin or ACE activity, or plasma Ang-II concentration. Injury-induced carotid artery intimal thickening also decreased significantly after dogs received NK3201 treatment [44]. These experiments suggest that chymase contributes to arterial wall thickening and neointima formation, likely by promoting inflammatory cell migration and

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vascular cell proliferation — a hypothesis that requires further investigation and confirmation.

In patients with atherosclerosis, serum total cholesterol or LDL cholesterol levels correlated with arterial chymase-dependent Ang-II forming activity [158]. Hamsters consuming a high-cholesterol diet developed lipid deposition in the aortic cusp. Plasma total or LDL cholesterol levels also correlated with adventitial Ang-II immunoreactivity. Oral administration of an orally active, non-peptide chymase inhibitor, SUN-C8257, completely suppressed aortic lipid deposition and Ang-II forming activity, although this inhibitor did not affect systolic blood pressure or serum total and LDL cholesterol levels [158, 159]. Similar observations were made in atherosclerosis-prone *Apoe*$^{-/-}$ mice [160]. A newly developed chymase inhibitor, RO5066852, reduced chymase activity and possibly tryptase activity; reduced spontaneous atherosclerosis; prevented local mast cell activation-induced acceleration of plaque progression; enhanced lesion collagen content and reduced lesion necrotic core size; and completely normalized the frequency and size of intraplaque hemorrhages in *Apoe*$^{-/-}$ mice after acute perivascular collar placement-induced carotid plaque [160].

CHYMASE INHIBITORS IN AAA

The best-studied chymase inhibitor in AAA is also NK3201. This inhibitor has been used in at least three experimental AAA models. In aortic elastase perfusion-induced experimental AAA in hamsters, treatment with NK3201 significantly reduced postelastase perfusion (2 weeks) aortic diameters (from 2.18±0.04 mm to 1.66±0.06 mm, $P < 0.01$), lesion tissue chymase activity (3.44±0.62 mU/mg to 2.03±0.31 mU/mg, $P < 0.05$), media thinning (media area to total area ratio from 41.3±6.7% to 68.4±7.5%, $P < 0.05$), and lesion mast cell numbers (14.3±1.3 cells/mm^2 to 8.58±0.9 cells/mm^2, $P < 0.01$) [41]. In the same experimental model, dogs receiving NK3201 showed significantly reduced aortic diameters 2–8 weeks post-elastase perfusion. NK3201 treatment decreased luminal area, increased media-to-total area percentage, reduced AAA lesion chymase-positive mast cells and neutrophils, and reduced lesion chymase activity, Ang-II forming activity, and MMP-9 activity [143].

When *Apoe*$^{-/-}$ mice receive systemic infusion of Ang-II, they develop suprarenal AAAs [161, 162]. Chymase inhibitor NK3201 suppressed lumen areas and arterial wall inflammatory cell (e.g., MOMA-2-positive monocytes and macrophages) infiltration and expansion in these mice. Mechanistically, in addition to inhibiting chymase activities, NK3201 also inhibited chymase-mediated MMP-9 activation in AAA lesions [144]. These animal experiments, as summarized in (Table **1**), support an essential role of mast cells and mast cell proteases in AAA pathogenesis, but whether chymase or tryptase can be drug targets for human AAA therapy remains unknown.

CHYMASE AND TRYPTASE INHIBITORS IN METABOLIC DISEASES

MC stabilizers are probably the best-studied anti-allergy drugs in metabolic disorders, and many are used to treat patients with allergies. Drugs in this category include cromolyn (Gastrocrom, or DSCG), ketotifen (Zaditor), and tranilast (Rizaben). Cromolyn and ketotifen are used in pediatric allergic disorders. Tranilast is used in bronchial asthma, atopic dermatitis, and allergic conjunctivitis, and recently has shown anti-angiogenic activity via inhibition of chymase and TGF-β [35]. Cromolyn [163] exerts its effect only on certain populations of mast cells, with limited effect on intestinal mucosal mast cells in rodents [164] and humans [165]. These effects are particularly important because cromolyn may inhibit mast cells in the connective tissues — such as those in the vasculature and WAT —

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without affecting the innate immunity of the mucosa against pathogens. In addition to its anti-allergy effect, ketotifen can control chronic inflammation processes such as autoimmune encephalomyelitis and arthritis [166], if given sufficient time points. Our most recent observations indicate that cromolyn and ketotifen can prevent diet-induced diabetes in mice. We also demonstrated that these mast cell stabilizers can reverse pre-established diabetes [93]; this result is particularly important for future clinical application. We need more comprehensive human trials to establish the applications of these mast cell drugs in patients; studies of mast cell protease inhibitors have just begun, however, and are still confined within animal models (Table **2**).

TEI-F00806 and TEI-E00548 are chymase-specific inhibitors recently developed by Teijin Pharma of Tokyo, Japan. In a multi-dose streptozotocin (30 mg/kg, one dose every 3 days for 2 weeks) treatment-induced diabetes model, hamsters develop diabetic nephropathy complicated by increased levels of blood glucose, Ang-II, urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG, a marker of oxidative stress), and urine protein (a marker of renal dysfunction). Kidneys from streptozotocin-treated mice show increased expression of chymase, ACE, Ang-II, NADPH oxidase components $p22^{phox}$ and NOX4 (NADPH oxidase 4), TGF-β1, and associated ECM protein fibronectin deposition. These kidneys showed increased oxidative stress and expansion of the glomerular mesangial area. Although TEI-F00806 and TEI-E00548 did not significantly change blood glucose levels or intrarenal ACE expression, both chymase inhibitors ameliorated all other aforementioned renal pathologies [109].

In a similar model of diabetes induced by a single high dose of streptozotocin (60 mg/kg), hamsters showed high blood glucose levels, increased pancreatic chymase, and Ang-II-forming activities. Mice receiving TY-51469, a chymase inhibitor from Toa Eiyo Co., Tokyo, Japan [167], significantly reduced blood glucose, chymase, total Ang II-forming activities, malondialdehyde, and mast cell numbers in pancreatic tissues, compared with those receiving placebo treatment. Furthermore, the TY-51469 group had significantly more pancreatic islets than the placebo group [110]. As summarized in (Table **2**), significant improvement in controls of blood glucose and kidney complications after treatment with different chymase inhibitors suggests a direct involvement of this mast cell protease — and possibly tryptase — in the pathogenesis of diabetes. Targeting these mast cell proteases may become a powerful means of managing metabolic diseases and their complications.

FUTURE PERSPECTIVES

Experimental diseases in animals have helped to test the role of mast cell-specific proteases in cardiovascular and metabolic diseases, although many current models do not fully recapitulate human diseases. Protease inhibitors are much more potent and selective than before, but we still lack clinical trials to test the efficacy of these potent and selective inhibitors in humans. Drug companies may be waiting for more mechanistic studies from chymase-deficient or tryptase-deficient animals, as these inhibitors may have off-target effects. For example, chymase inhibitor RO5066852 also targeted cathepsin G, although IC50 is 27-fold higher than chymase [160]. Direct approvals in protease-deficient animals may help to advance the progress of proposing human trials.

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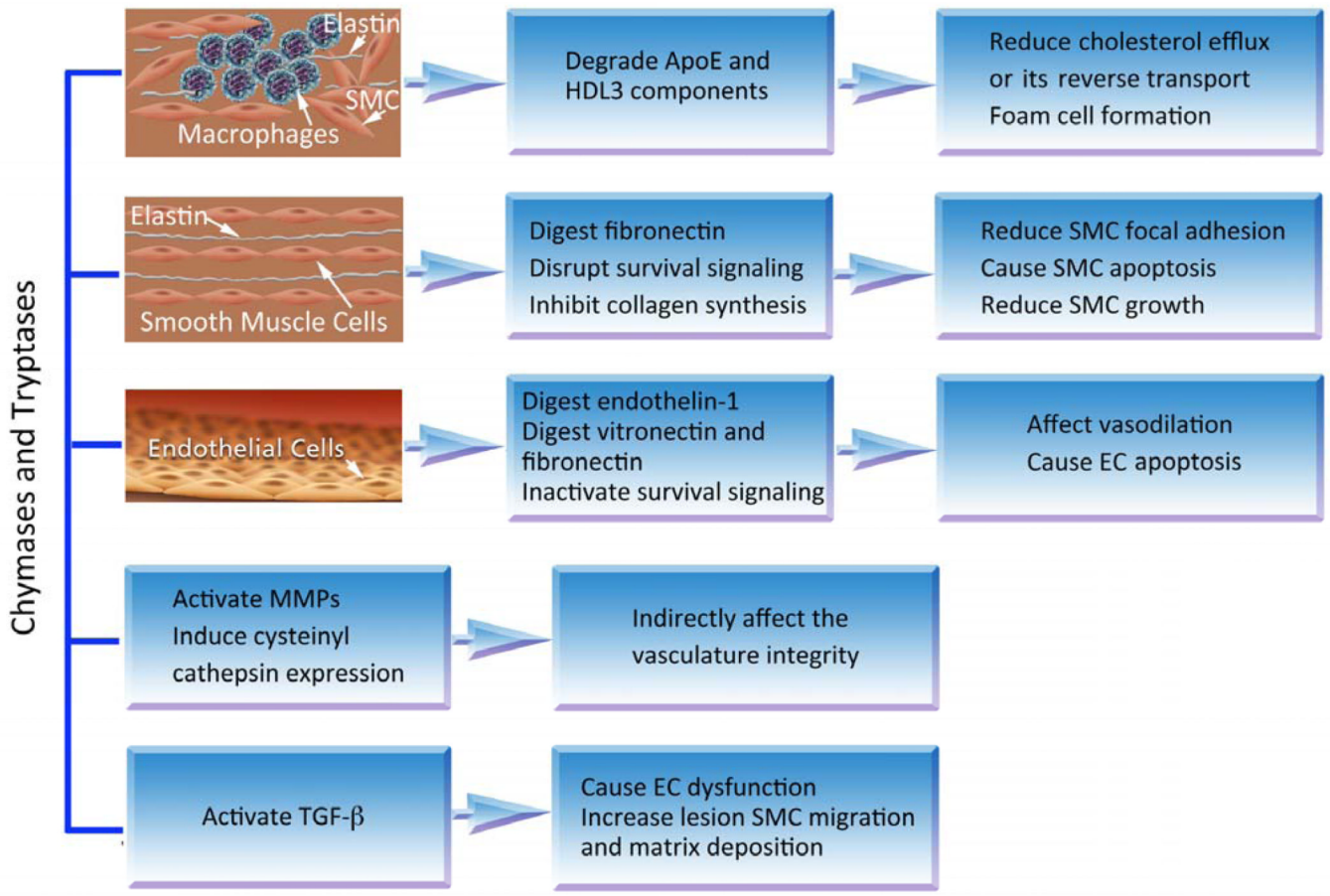

Fig. (1).

Role of mast cell chymase and tryptase in atherogenesis. Besides acting on macrophages, SMCs, and ECs, these proteases also regulate the expression and activation of other proteases (MMPs and cysteinyl cathepsins) or growth factors (e.g., TGF-β), thereby indirectly affecting atherogenesis.

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Table 1

Chymase and Tryptase Inhibitors in Cardiovascular Diseases

Inhibitor (inhibitor category) [Experimental model] (reference)	**Major findings**	**Mechanisms**
NK3201 (chymase inhibitor) [Dog carotid artery bypass /injury neointima models] [44, 157] [Elastase-induced AAA in dog and hamster] [41, 143] [Ang-II perfusion-induced AAA in *Apoe*$^{-/-}$ mice] [144]	Reduced neointimal thickening Reduced aortic diameter, media thinning and luminal area Reduced arterial wall infiltration of mast cells, neutrophils, monocytes, and macrophage	Reduced lesion chymase activity and associated Ang-II forming activity and MMP activity Reduced stem cell factor activation Reduced lesion vascular cell proliferation and inflammatory cell infiltration
R05066852 (chymase inhibitor) [*Apoe*$^{-/-}$ carotid plaque model] [160]	Reduced atherosclerotic plaque size and progression Reduced lesion necrotic core size Enhanced lesion collagen content Reduced intraplaque hemorrhage frequency and size	Reduced chymase activity and possibly tryptase activity
SUN-C8257 (chymase inhibitor) [Hamster diet-induced atherosclerosis model] [158, 159]	Suppressed aortic lipid deposition in hamster atherosclerotic lesions No effect on blood pressure or blood cholesterols	Suppressed Ang-II forming activity
APC-366 (tryptase inhibitor) [Cultured human macrophages] [156]	Reduced numbers of separated lipid vacuoles in human macrophages Reduced intracellular neutral lipid in human macrophages	Increased ABCG1/ABCA1/SREBP-1c expression and blocked reduction of nuclear receptor LXRa expression in human macrophages, in addition to tryptase activity inhibition

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Table 2

Chymase Inhibitors in Diabetes

Inhibitor [Experimental model] (reference)	**Major findings**	**Mechanisms**
TEI-F00806 TEI-E00548 [Multi-dose streptozotocin-induced diabetes in hamster] [109]	Reduced proteinuria and systemic oxidative stress Reduced glomerular fibronectin deposition Reduced glomerular mesangial expansion No effect on blood glucose	Reduced kidney expressions of chymase, Ang-II, NADPH oxidase components, TGF-β1
TY-51469 [Multi-dose streptozotocin-induced diabetes in hamster] [110]	Attenuated blood glucose level Reduced pancreas oxidative stress Increased pancreatic islets	Reduced pancreas chymase and Ang-II forming activities Reduced pancreas mast cell number