*Current Perspective***Multiple Mechanisms for the Action of Chymase Inhibitors**Shinji Takai^{1,*}, Denan Jin¹, and Mizuo Miyazaki¹¹Department of Pharmacology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki City, Osaka 569-8686, Japan

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Abstract. Angiotensin II plays an important role in regulating blood pressure. Moreover, angiotensin II directly promotes organ damage by inducing expression of various genes, such as transforming growth factor (TGF)- β and matrix metalloproteinase (MMP)-9 precursors. Blockade of angiotensin II has been shown to not only lower blood pressure, but also to prevent cardiovascular and renal dysfunction and fibrosis. Inhibition of TGF- β and MMP-9 has also been shown to prevent cardiovascular and renal damage. A mast cell-produced enzyme, chymase, generates angiotensin II and also converts precursors of TGF- β and MMP-9 to their active forms. Chymase also strongly promotes accumulation of inflammatory cells. These multiple functions of chymase may play an important role in the development and promotion of various diseases. In fact, chymase inhibitors have been shown to prevent nonalcoholic steatohepatitis, intestinal inflammation, and adhesion formation after surgery and cardiovascular and renal damage. On the other hand, chymase inhibitors, unlike angiotensin-converting enzyme inhibitors and angiotensin II blockers, have no blood pressure-lowering effect despite blocking angiotensin II formation. Thus, chymase inhibitors may be useful for preventing damage to various organs via multiple mechanisms without lowering blood pressure.

Keywords: angiotensin II, chymase, inhibitor, matrix metalloproteinase-9, transforming growth factor- β

1. Introduction

Chymase, like angiotensin-converting enzyme (ACE), is known as an angiotensin II-forming enzyme. In homogenates from human vascular tissues, approximately 90% of angiotensin II-forming activity is dependent on chymase, and the remaining requires ACE (1). Angiotensin II induces vascular constriction and induces a pressor response. Chymase inhibitors reduce chymase-dependent angiotensin II formation in homogenate from vascular tissues, and one may assume that chymase inhibitors, like ACE inhibitors and angiotensin II-receptor blockers (ARBs), are useful for lowering blood pressure. However, blood pressure was not lowered by chymase inhibitors (2). Furthermore, ACE inhibitors and ARBs increase plasma renin activity, while chymase inhibitors

do not (2). These findings suggest that chymase inhibitors, but not ACE inhibitors and ARBs, may have little involvement in the regulation of the systemic renin-angiotensin (RA) system (Fig. 1).

When evaluating chymase inhibitors as angiotensin II inhibitors, species differences should be considered. Chymases in humans, monkeys, dogs, mice, swine, and hamsters show angiotensin II-forming ability, but chymase in rats does not (2 – 4). This difference may be very important information. In rats, neointimal formation in arteries after balloon catheter injury was significantly attenuated by both ACE inhibitors and ARBs (5, 6). However, in the dog balloon injury model, the neointimal formation was significantly attenuated by an ARB, but not by an ACE inhibitor (7). Moreover, a chymase inhibitor showed significant attenuation of the neointimal formation in dogs (8). In clinical studies, restenosis after percutaneous coronary intervention (PCI) was prevented by an ARB, but it was not by an ACE inhibitor (9, 10). Thus, species differences are thought to be very important in the evaluation of chymase inhibitors. On the other hand, chymase has additional functions, such as activat-

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Fig. 1. Chymase is stored as an inactive enzyme in secretory granules in which chymase has no enzymatic activity. Chymase activity shows almost a maximum level immediately upon release into the tissues when mast cells are stimulated. ACE always functions as an angiotensin (Ang) II-forming enzyme. The tissue RA system is dependent on ACE and chymase pathways and the circulating RA system is dependent on ACE pathway.

ing transforming growth factor (TGF)- β and matrix metalloproteinase (MMP)-9 precursors (2). Unlike its angiotensin II-forming ability, these functions of chymase are shown in all species including rats. Therefore, chymase inhibitors have multiple mechanisms that need to be considered for each target disease without focusing solely on species differences.

2. Effects of chymase inhibitor in hypertension

Both angiotensin II-forming enzymes, ACE and chymase, are expressed in vascular tissues, but ACE is mainly expressed in vascular endothelial cells, and chymase is stored in secretory granules of mast cells in the adventitia. ACE localized in vascular endothelial cells always exerts its angiotensin II-forming capability, but chymase may not do so because it is stored in the secretory granules in mast cells, where it has no enzymatic functions. Chymase is stored in the secretory granules, where the pH is maintained at 5.5. The pH optimum of chymase is between 7 and 9, and chymase activity has no enzymatic activity at pH 5.5. Chymase must exert its enzymatic activity when it is released into the interstitial tissues from mast cell granules due to the stimulation that occurs from injury or inflammation. On the other hand, in human vascular extracts, over 90% of the angiotensin II formation was inhibited by a chymase inhibitor (1). This

finding suggests that over 90% of the angiotensin II formation in human vascular tissue extracts is dependent on chymase. Chymase ordinarily has no enzymatic activity, including angiotensin II formation, *in situ*, while in the extracts, chymase-dependent angiotensin II-forming activity may potentially be high (2). Moreover, in blood, unlike ACE, chymase has no enzymatic ability because the enzymatic ability of chymase is immediately abolished by internal serine protease inhibitors. Therefore, chymase-dependent angiotensin II formation occurs only in injured and damaged tissues.

Species differences should be considered when discussing chymase-dependent angiotensin II formation as one mechanism of action of chymase inhibitors. Although angiotensin II formation was completely suppressed by an ACE inhibitor in rat vascular extract, it was partially inhibited in human vascular extract (2). The extracts from rat vascular tissues also contain rat chymase, which produces hardly angiotensin II. The reason why human chymase, but not rat chymase, can form angiotensin II from angiotensin I is due to differences in the substrate specificity of these chymases. Using the substrate angiotensin I, human chymase does cleave the Phe⁸-His⁹ bond of angiotensin I to yield angiotensin II, while rat chymase cleaves the Tyr⁴-Ile⁵ bond to form inactive fragments (2). Therefore, rat models may be irrelevant when examining chymase-dependent angiotensin II formation.

The RA system plays an important role in the regulation of blood pressure. However, it has been unclear whether chymase-dependent angiotensin II formation is involved in regulating blood pressure; therefore, we studied a 2K1C hypertensive hamster model with an angiotensin II-forming chymase (11). Plasma renin and vascular ACE activities, but not vascular chymase activity, increased significantly in this model. ACE inhibitors and ARBs are known to not only reduce blood pressure but also to increase plasma renin activity, while chymase inhibitors affected neither blood pressure nor plasma renin activity (2). These findings suggest that chymase inhibitors, but not ACE inhibitors and ARBs, may be little involved in the regulation of blood pressure. Therefore, chymase inhibitors may be inadequate as antihypertensive agents.

3. Effects of chymase inhibitor in ischemic heart diseases

The onset of myocardial infarction is closely related to hypertension. Although chymase inhibitors may be inadequate as antihypertensive agents, they may be useful for preventing the onset of myocardial infarction. The development of acute myocardial infarction is mainly dependent on plaque rupture in coronary arteries, and mast

cells containing chymase accumulate more at ruptured sites than at normal regions (12). MMP-9 was highly expressed in the ruptured sites, and it is thought to be involved in thinning of the fibrous cap and eventual rupture (12). Indeed, multiple logistic regression analysis showed that MMP-9 level was the only independent predictor of plaque rupture (12). In extracts from human vascular tissues, a chymase inhibitor significantly reduced MMP-9 formation from proMMP-9 (13). Therefore, chymase inhibitors may be useful for preventing the onset of myocardial infarction via reduction of the active form of MMP-9.

Chymase is activated in the ischemic myocardium after ligation of the coronary artery in animal models (3, 14). The elevated chymase activity has been found to augment angiotensin II formation and MMP-9 activity, while chymase inhibitors attenuated angiotensin II formation and MMP-9 activity (3, 14). Chymase inhibitors attenuated the cardiac dysfunction and extended survival (3, 14, 15). Attenuation of cardiac dysfunction and extension of survival were also observed with ARB treatment and in MMP-9 null mice after myocardial infarction (16). Therefore, angiotensin II suppression and MMP-9 inhibition by chymase inhibitors may be considered useful for suppressing cardiac dysfunction and mortality after myocardial infarction.

Not only human, dog, and hamster chymases, but also rat chymase activates the precursor of TGF- β to its active form (2). TGF- β is a major stimulator of myocardial fibrosis and is closely involved in the promotion of cardiac fibrosis. To clarify the role of chymase-dependent TGF- β activation, we evaluated whether chymase inhibition prevents cardiac fibrosis and cardiac dysfunction after myocardial infarction in rats that have no angiotensin II-forming chymase in their hearts (17). Chymase activity was significantly increased after myocardial infarction, but it was significantly reduced by chymase inhibitor treatment (17). Both the fibrotic area and the mRNA levels of collagen I and collagen III were also significantly decreased by the chymase inhibitor treatment. These results suggest that a chymase inhibitor may also prevent cardiac dysfunction after myocardial infarction without reducing angiotensin II activity. In the rat model, the pathway of chymase-dependent TGF- β activation may also play an important role in myocardial fibrosis and cardiac dysfunction in ischemic myocardium.

Although a chymase inhibitor, but not ACE inhibitor and ARB, may not directly lower blood pressure in patients with hypertension, it may be useful for preventing the onset of ischemic heart diseases or progression of cardiac dysfunction after myocardial infarction.

4. Effects of chymase inhibitors in abdominal aortic aneurysm

Hypertension and atherosclerosis are well known to be involved in the development of abdominal aortic aneurysms (AAAs). The involvement of increased angiotensin II in the development of AAA may go beyond its pressor effects. A previous paper demonstrated that both 250 and 1000 ng/kg per min of angiotensin II infusion given to apoE-deficient mice resulted in a maximum blood pressure increase of approximately 40 mmHg (18). However, AAA development was observed with a dose of 1,000 ng/kg per min of angiotensin II, but not with a dose of 250 ng/kg per min (18). In contrast, hydralazine lowered blood pressure, but it did not attenuate AAA formation in angiotensin II-infused apoE-deficient mice (19). Therefore, AAA development may be independent of the blood pressure, and angiotensin II may be closely involved in AAA development.

AAAs represent a chronic degenerative condition associated with atherosclerosis, characterized by segmental weakening and dilatation of the aortic wall. The pathophysiology of AAA includes aortic inflammation within the outer aortic wall and an imbalance between the production and degradation of structural extracellular matrix proteins. Chymase may be involved in the pathogenesis of AAAs, because chymase converts proMMP-9 to MMP-9, which has the highest affinity for elastin as a substrate; it is thus considered to play an important role in the pathology of AAA (11). Angiotensin II stimulation induces proMMP-9 gene expression, and its function may be important for AAA progression. In fact, both chymase and ACE activities were significantly increased when the extract was incubated with an extract of aorta from patients with AAAs (4). These observations suggest that proMMP-9 gene expression is strongly induced by ACE- and chymase-dependent angiotensin II formation and that chymase also plays an important role in the activation of proMMP-9 to MMP-9 in extract of aorta from patients with AAAs.

In angiotensin II-infused apoE-deficient mice, a significant expansion of aortic diameter was observed, along with significant augmentation of ACE, chymase, and MMP-9 activities in the aorta (20). With chymase inhibitor treatment, not only chymase activity but also MMP-9 activity was significantly attenuated, along with attenuation of AAA development (20). On the other hand, a significant blood pressure increase was observed in angiotensin II-infused apoE-deficient mice, but it was not changed with chymase inhibitor treatment. In this model, proMMP-9 expression was induced by angiotensin II, and the target of chymase inhibitors might be downstream of angiotensin II action, such as inhibition of MMP-9

activation. Thus, the mechanism of action of chymase inhibitors for preventing AAA progression might involve not only the inhibition of angiotensin II formation but also the inactivation of proMMP-9 (Fig. 2).

5. Effects of chymase inhibitors in diabetes

Augmentation of angiotensin II stimulation may be related to not only the development of diabetes but also to the pathogenesis of diabetic complications (21). Blockade of angiotensin II is associated with increases in insulin-stimulated skeletal muscle glucose transport and the glucose transporter GLUT-4 in skeletal muscle. Furthermore, ACE inhibitors and ARBs attenuate the changes in pancreatic structure and function that are related to β -cell dysfunction. Angiotensin II promotes the induction of NADPH oxidase expression, increasing reactive oxygen species that promote pancreatic islet disorganization. Therefore, the attenuation of pancreatic islet disorganization by angiotensin II blockade may also contribute to the reduced incidence of new-onset and progression of diabetes.

Chymase may be involved in the progression of diabetes. In hamsters, both chymase and angiotensin II-forming activities were significantly increased in pancreas extract along with blood glucose levels after streptozotocin injection (22). A chymase inhibitor significantly attenuated not only the pancreatic chymase and total angiotensin II-forming activities but also the blood glucose levels. In this model, a marker of oxidative stress, malondialde-

hyde, was significantly increased in the pancreas, but this level was significantly reduced by a chymase inhibitor. After streptozotocin injection, β -cell expression in the pancreas was dramatically decreased, but it was significantly maintained in hamsters treated with a chymase inhibitor (22). Thus, the mechanism of action of chymase inhibitors may be closely involved in the reduction of angiotensin II-induced oxidative stress in the pancreas, resulting in protection of β -cell function. On the other hand, TGF- β also contributes to the progression of diabetic complications (23). In the rat diabetic model, anti-TGF- β antibody showed a significant preventative effect against renal damages (23). Furthermore, combination treatment with anti-TGF- β antibody and ACE inhibitor showed more powerful protective effects against renal damage (23). Thus, the inhibition of chymase-dependent TGF- β activation, in addition to the suppression of angiotensin II formation, may be one mechanism through which chymase inhibitors prevent diabetic pathogenesis and organ damages.

6. Effects of inflammation-induced organ damages

Chymase functioned as a potent chemoattractant and that it could induce chemotactic migration of monocytes and neutrophils (24). Both macrophages and neutrophils strongly express proMMP-9, and chymase inhibition may result in reduction of proMMP-9 levels, in addition to inhibition of MMP-9 activation. Therefore, chymase inhibitors may be useful for prevention of inflammation-related diseases.

Nonalcoholic steatohepatitis (NASH) is accompanied by metabolic syndrome, which consists of obesity, diabetes, and hypertension. In patients with NASH, progressive steatosis, lobular inflammation, and fibrosis have been observed in the liver. In a hamster NASH model, the levels of the biochemical markers total bilirubin, hyaluronic acid, and triglycerides, all of which are known to increase in patients with NASH, were significantly increased (25). In addition, marked steatosis and fibrosis on histological analysis were also observed in the hamster NASH model. However, all of the biochemical markers and histological changes were significantly attenuated by treatment with a chymase inhibitor. In this model, chymase inhibition showed a significant reduction in angiotensin II-forming and MMP-9 activities, both of which are involved in the steatosis and fibrosis in the liver.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been widely used for their anti-inflammatory effects, but they are known to produce gastric damage. After administration of indomethacin, MMP-9 activity was augmented, and the upregulation of MMP-9 induced intestinal barrier dysfunction and bacte-

Fig. 2. Angiotensin II augments MMP-9 and TGF- β gene expressions. Inhibition of angiotensin II action by ACE inhibitor and chymase inhibitor results in the reduction of MMP-9 and TGF- β gene expressions. ARB also decreases MMP-9 and TGF- β gene expressions via blockade of angiotensin II receptor. Chymase converts precursor TGF- β and proMMP-9 to TGF- β and proMMP-9, respectively, and chymase inhibitor inhibits these activations.

rial translocation (26). In the rat indomethacin-induced small intestinal injury model, a chymase inhibitor reduced MMP-9 activity in the small intestine, preventing small intestinal damage (27). Myeloperoxidase activity, which indicates neutrophil accumulation, was significantly increased in the small intestine after indomethacin administration, but its activity was significantly attenuated by treatment with a chymase inhibitor. Neutrophils express proMMP-9, and chymase inhibition may result in attenuating not only MMP-9 activity but also proMMP-9 expression levels. The effects of chymase inhibitors related to the prevention of indomethacin-induced small-intestinal damage were observed in rats, and this mechanism of action of chymase inhibitors may be unrelated to the inhibition of angiotensin II.

Postoperative adhesions are a well-known complication of surgery and may involve the accumulation of inflammatory cells including mast cells. The number of mast cells is increased around wounds in the late stages of the healing process. In a mouse adhesion model, the TGF- β level in peritoneal fluid was significantly higher after surgery, but intraperitoneal injection of a neutralizing antibody to anti-TGF- β decreased adhesion formation (28). In hamster and rat adhesion models, chymase activity was significantly increased in the region of the adhesion, while it was significantly reduced by treatment with chymase inhibitors, along with reduction in adhesion formation (29, 30). TGF- β concentrations in the pleural fluid were significantly increased after cardiac surgery, while the increased TGF- β concentrations were significantly reduced by treatment with chymase inhibitors (29, 30). Thus, chymase inhibitors may become a useful strategy for prevention of postoperative adhesions via TGF- β reduction, but not angiotensin II reduction.

Angiotensin II augments gene expressions of MMP-9 and TGF- β , and blockade of angiotensin II function using ACE inhibitor and ARB results in the prevention of MMP-9- and TGF- β -related diseases such as cardiovascular dysfunction and tissue fibrosis including NASH (Fig. 2). However, other factors such as inflammation also contribute to the augmentation of MMP-9 and TGF- β gene expressions. Chymase inhibitor inhibits activations of TGF- β precursor and proMMP-9 in addition to angiotensin II formation (Fig. 2). Chymase inhibitor may attenuate their actions induced by factors other than angiotensin II stimulation.

7. Conclusion

Angiotensin II-blocking agents such as ACE inhibitors and ARBs have been widely used in preventing cardiovascular and renal dysfunction, as well as lowering blood pressure. Chymase is known to increase tissue angio-

Fig. 3. ACE inhibitor and ARB are useful for prevention of angiotensin-related diseases, but a chymase inhibitor may be useful for prevention of angiotensin II- and inflammation-related diseases.

tensin II levels, but chymase inhibitors may be considerably different from RA system-related inhibitors, ACE inhibitors, ARBs, and direct renin inhibitors. In general, conventional RA system-related inhibitors have been used to treat patients with hypertension. Although angiotensin II blockade may prevent organ damage in non-hypertensive patients, the RA system-related inhibitors show hypotensive effects, and these agents are rarely used for normotensive patients. On the other hand, chymase inhibitors may attenuate angiotensin II formation only in damaged organs, and they may not show hypotensive effects. Moreover, chymase inhibitors have shown various useful effects that are independent of angiotensin II inhibition. We propose that chymase inhibitors may be widely used for prevention of angiotensin II- and inflammation-related diseases (Fig. 3).

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