Forum Minireview

**Physiology and Pathophysiology of Proteinase-Activated Receptors (PARs):
Role of Trypsin/PAR-2 in Vascular Endothelial Barrier Function**Yoshinori Itoh^{1,*}, Toshiaki Sendo¹, and Ryozi Oishi¹¹Department of Pharmacy, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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Abstract. Proteinase-activated receptor-2 (PAR-2) plays important roles in a variety of pathophysiological functions, including inflammatory responses and nociception. In this minireview, we describe the role of PAR-2 in acute inflammatory responses in lungs associated with iodinated radiographic contrast medium (RCM). Intravenous injection of RCM to rats induces lung injury characterized by vascular hyperpermeability, edema, and respiratory depression. Nafamostat, which is found to be the most potent and specific trypsin inhibitor, prevents RCM-induced lung injury. In cultured endothelial cells of human pulmonary artery and bovine aorta, RCM, when applied in combination with mast cells, disrupts barrier function evaluated by the permeability of Evans blue through a monolayer of cultured cells, which is blocked by nafamostat and mimicked by trypsin and PAR-2-activating peptide. The trypsin-induced barrier dysfunction is blocked completely by a phospholipase C inhibitor and partially inhibited by a IP₃ receptor blocker, protein kinase C inhibitor, or Rho kinase inhibitor. Morphological observations reveal the formation of actin stress fibers and disappearance of the intercellular meshwork structure of vascular endothelial-cadherin after application of RCM or PAR-2 ligands. Therefore, the release of mast cell trypsin and subsequent activation of endothelial PAR-2 are involved in acute lung injury induced by RCM.

Keywords: mast cell trypsin, nafamostat, radiographic contrast medium, vascular permeability, proteinase-activated receptor-2

Nafamostat is the most potent and specific trypsin inhibitor

Trypsin is a serine protease that is included almost exclusively in mast cell granules (1). Mast cell trypsin is a potent modulator of microvascular leakage and inflammatory responses during episodes of allergic reactions (2). The concentration of trypsin is elevated in the plasma of asthmatic patients after allergen challenge (3, 4) and in the nasal lavage fluid of allergic patients (5).

On the other hand, allergy-like adverse reactions including skin rash, blushing, and urticaria occur immediately after intravascular injection of radiographic contrast medium (RCM) (6). In severe cases, RCM causes bronchospasm, dyspnea, and pulmonary edema (7). The RCM-induced pulmonary edema is a non-cardiogenic type and appears to be related to the increase

in pulmonary vascular permeability due to the activation of the inflammatory cascade and the release of a variety of chemical mediators (8). It has been demonstrated that the plasma level of trypsin is elevated in patients who showed hypersensitivity reactions after intravascular injection of RCM (9, 10). However, the role of mast cell trypsin in the pathogenesis of the anaphylactoid reactions to RCM remains to be clarified because of the lack of any available specific trypsin inhibitors.

Recently, Erba et al. (11) have reported that gabexate is a highly potent and specific inhibitor of human trypsin. This compound is a synthetic low molecular-weight inhibitor of trypsin-like serine proteases (12–14), but shows more than 100-fold higher affinity for human trypsin than for other proteases such as thrombin and trypsin. Gabexate is used clinically for the therapy of acute pancreatitis and disseminated intravascular coagulation in Japan.

We confirmed that gabexate inhibits the activity of

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human lung tryptase. More importantly, nafamostat, another structurally related serine protease inhibitor, was found to be far more potent than gabexate. In purified human tryptase, both gabexate and nafamostat inhibit tryptase activity in a concentration-dependent manner, in which IC_{50} values are 1.9×10^{-7} M for gabexate and 1.6×10^{-11} M for nafamostat (15). The mode of tryptase inhibition by these compounds is competitive (16). Moreover, the affinity of nafamostat for human tryptase is 157- and 8814-fold higher than those for trypsin and thrombin, respectively (16). Therefore, the compound appears to be useful for the research on the physiological roles of tryptase, although the biological half-life of this compound is short (23.1 min) (17).

Role of tryptase in RCM-induced acute lung injury in rats

We have already found in rats that RCM causes extravasation of plasma proteins in lungs, pulmonary edema (18, 19), and respiratory dysfunction characterized by the decrease in arterial PaO_2 and increase in $PaCO_2$ (20, 21) immediately (within 10 min) after intravenous injection. These actions of RCM are partially blocked by histamine H_1 - and H_2 -receptor antagonists, while markedly attenuated by cromoglycate, a mast cell stabilizer, and depletion of mast cells by repeated injection of compound 48/80 (22). Therefore, the RCM-induced acute lung injury may be mediated by mast cell ingredients. Indeed, RCM stimulates the release of pre-formed chemical mediators such as histamine (23–26) and tryptase (21, 27, 28) from mast cells without affecting the release of newly synthesized mediators such as leukotrienes B_4 and C_1 , or prostaglandin D_2 (29, 30).

On the other hand, plasma extravasation in rat lungs induced by the intravenous injection of RCM is markedly reduced by nafamostat and slightly inhibited by gabexate (Fig. 1A). Nafamostat also prevents RCM-induced pulmonary edema (Fig. 1B) as well as the respiratory dysfunction (Fig. 1C). Therefore, it is suggested that mast cell tryptase contributes to the pathogenesis of acute lung injury induced by RCM.

Regulation of barrier function by endothelial PAR-2

Several lines of evidence have suggested that some of physiological actions of tryptase are mediated by the stimulation of proteinase-activated receptor (PAR)-2 (31, 32). PAR-2 is activated by its tethered ligand after cleavage of Arg-Ser bond near the N-terminal region by tryptase, trypsin, factor VIIa, and factor Xa (33, 34). The activation of PAR-2 results in the generation of nocicep-

tive signals and pain (35), increase in airway resistance (36), and enhancement of vascular permeability (37, 38). Kawabata et al. (37) originally reported that PAR-2-activating peptide (PAR-2AP) causes enhancement of vascular permeability and edema in rat hind paws. On the other hand, Steinhoff et al. (39) have shown that the vascular action of PAR-2AP in rat hind paws is due to the release of sensory peptides such as calcitonin gene-related peptide and substance P from sensory nerve endings. Indeed, PAR-2 is expressed in sensory nerves, and activation of this receptor stimulates the release of sensory peptides (39). Although both PAR-2 mRNA and its protein are highly expressed in vascular endothelial cells (40, 41), it is still uncertain whether endothelial PAR-2 directly regulates the barrier function.

In cultured monolayer of bovine aortic endothelial cells (BAEC) (15) or human pulmonary arterial endothelial cells (HPAEC) (21), RCM causes a marked and concentration-dependent increase in the permeability of proteins, when it is applied in combination with mast cells. The RCM-induced increase in endothelial permeability is suppressed by cromoglycate and nafamostat (15), thereby suggesting that the barrier dysfunction is mediated predominantly by mast cell tryptase. Indeed, tryptase causes a marked and concentration-dependent increase in endothelial permeability in BAEC (Fig. 2A), and this action is inhibited by nafamostat and gabexate in a concentration-dependent manner (Fig. 2B). In addition, the endothelial permeability is enhanced by PAR-2AP such as SLIGKV-NH₂ but not its reverse peptide LSIKGV-NH₂ (Fig. 2A). Taken together, it is highly probable that RCM-induced barrier dysfunction is due to the activation of endothelial PAR-2.

In contrast to our data, it has been reported that PAR-2 stimulation does not affect barrier function in cultured human umbilical vein endothelial cells (42, 43). Although the reason for such a discrepancy is not known, differences in blood vessels (aorta and vein) or species may cause such a different role of PAR-2 in endothelial barrier function.

Intracellular signaling involving PAR-2-mediated increase in permeability through monolayer of cultured endothelial cells

It has been demonstrated that activation of PAR-2 stimulates phosphatidylinositol hydrolysis and results in the elevation of $[Ca^{2+}]_i$ in numerous types of cells (33, 44) including endothelial cells (45), in which PAR-2 is coupled with G proteins G_q/G_{11} (33). In BAEC, tryptase or PAR-2AP causes a marked elevation of $[Ca^{2+}]_i$ and desensitization occurs after repeated stimulation (15). The PAR-2-mediated increase in endo-

Fig. 1. Inhibition by nafamostat and gabexate of the vascular hyperpermeability in lungs (A), pulmonary edema (B), and respiratory dysfunction (C) induced by the intravenous injection of RCM in rats. Respiratory dysfunction is assessed by monitoring arterial gases. * $P < 0.05$, ** $P < 0.01$ vs control; † $P < 0.05$ vs ioxaglate alone. Data from Sendo et al. (Ref. 21)

thelial permeability is reversed almost completely by the phospholipase C (PLC) inhibitor U73122 and partially inhibited by the protein kinase C (PKC) inhibitor calphostin C, the 1,4,5-trisphosphate (IP₃)-receptor antagonist xestospongine C, or the Rho kinase inhibitor Y27632 (15). Therefore, activation of PKC and Rho kinase and elevation of [Ca²⁺]_i may contribute to PAR-2-mediated endothelial barrier dysfunction.

Formation of actin stress fibers and disappearance of VE-cadherin structure after PAR-2 activation in cultured endothelial cells

The endothelial barrier function is regulated by the intercellular tight junctional organization. Vascular endothelial (VE)-cadherin is the major constituent of cell-to-cell contact and interacts with actin filaments

via associated proteins such as α - and β -catenins (46). The VE-cadherin-catenin complex is flexible and disappears from the extracellular surface in response to various stimuli such as thrombin that disrupt endothelial barrier function (47). The gap formation due to the formation of actin stress fibers and its interaction with myosin is also involved in endothelial barrier dysfunction (48). Stimulation of BAEC with tryptase or PAR-2AP causes disappearance of immunoreactive VE-cadherin with a marked development of actin stress fiber (15, 21). RCM, when applied in combination with mast cells, also results in similar change in VE-cadherin immunoreactivity and actin stress fiber formation (15, 21). It has been reported that stimulation of PAR-2 results in the rapid activation of RhoA and subsequent formation of actin stress fibers in prostate cancer cell line LNCaP cells (49) as well as in endothelial cells (50).

Fig. 2. Enhancement of permeability of BAEC by tryptase, trypsin, and a synthetic PAR-2-activating peptide SLIGKV-NH₂ but not its reverse peptide LSIQKV-NH₂ (A) and reversal by nafamostat and gabexate of tryptase-induced increase in endothelial permeability (B). Data from Sendo et al. (Ref. 15)

Fig. 3. Schematic drawing for possible cellular mechanisms underlying endothelial barrier dysfunction induced by radiographic contrast medium (RCM). RCM causes degranulation of mast cells and stimulates the release of tryptase, which in turn, activates endothelial PAR-2. The elevation of intracellular Ca²⁺ and activation of PKC after PAR-2 stimulation may trigger the formation of actin stress fibers and disappearance of tight junctional proteins such as VE-cadherin, which leads to barrier dysfunction. GDI: guanine nucleotide dissociation inhibitor, MLCK: myosin light chain kinase.

Since PAR-2-mediated increase in permeability through a monolayer of BAEC is attenuated by Y27632, the activation of RhoA/Rho kinase may play a role in PAR-2-mediated gap formation.

Conclusion

We demonstrated for the first time that the release of mast cell tryptase and subsequent activation of PAR-2 are implicated in the pathogenesis of acute lung injury induced by RCM. Moreover, the endothelial PAR-2 regulates directly the barrier function in some arteries. The cellular mechanisms underlying RCM-induced endothelial barrier dysfunction is summarized in Fig. 3. RCM is a potent secretagogue of mast cell substances such as histamine and tryptase. The released tryptase activates endothelial PAR-2 and results in the formation of IP₃ and diacylglycerol via activation of PLC. Both PKC stimulated by diacylglycerol and elevation of [Ca²⁺]_i via stimulation of IP₃ receptor are involved in endothelial barrier dysfunction, in which Rho kinase-dependent gap formation and disappearance of VE-cadherin structure are involved.

In this respect, the potent and selective tryptase inhibitor nafamostat may be potentially useful for prophylaxis of severe pulmonary injury associated with radiographic examination in high-risk patients.

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