Enzymatic Measurement of Tryptase-Like Protease Release from Isolated Perfused Guinea Pig Heart during Ischemia-Reperfusion

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To elucidate the details of tryptase release from the heart during ischemia-reperfusion (I/R), we attempted the enzymatic measurement of tryptase release from the isolated guinea pig heart perfused by the Langendorff mode I/R model. Tryptase-like activity in the effluent was monitored by the hydrolysis of L-Pyr-Gly-Arg-MCA. Tryptase-like protease and histamine were rapidly released from heart during ischemia within 10 min. After reperfusion, tryptase-like protease levels decreased, achieving stabilization. The tryptase-like protease activity in the effluent was inactivated by serine protease inhibitors. The pattern of inhibition was similar to those of guinea pig and human lung tryptase. In conclusion, tryptase was released into the coronary effluent during ischemia, but not during reperfusion in guinea pig heart.

Key words tryptase; guinea pig; heart; ischemia-reperfusion

Large numbers of mast cells have been identified in the heart in animals and in humans. However, the role played by the mast cells in the heart has remained largely elusive. In order to clarify this role, a number of researches has attempted to determine the involvement of histamine and other chemical mediators released from heart mast cells.¹⁾ Heart mast cells have been shown to release histamine as a result of degranulation by ischemia-reperfusion (I/R),²⁾ and histamine and other chemical mediators released from heart mast cells play various roles in myocardial I/R injury.³⁾ Mast cells can also release various proteases (*e.g.*, tryptase, chymase, and carboxypeptidase) on degranulation.⁴⁾ Tryptase, the most abundant neutral mast cell protease. However, the details of tryptase release during I/R in the heart, remain unclear. In the present study, we attempted the enzymatic determination of tryptase release from the isolated guinea pig heart using the Langendorff mode I/R model.

MATERIALS AND METHODS

Animals All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. Female Hartley guinea pigs (Japan SLC, Inc., Hamamatsu) weighing 375–430 g were anesthetized with pentobarbital sodium (30 mg/kg *i.p.*).

Experimental Model The hearts were rapidly excised from the thoracic cavity, the aorta was cannulated, and the hearts were then transferred to a Langendorff apparatus (AD Instruments Pty. Ltd., NSW, Australia). The hearts were retrogradely perfused at a constant pressure of 40 mmHg with Krebs–Henseleit (K-H) buffer containing (mM): NaCl, 118.1; KCl, 4.8; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; and glucose, 11.1. In the measurement of creatine phosphokinase (CPK) and histamine releases, 50 μ M of atenolol and 10 μ M of SKF-91488 (Tocris Cookson Inc., Ellisville, MO, U.S.A.), a histamine *N*-methyltransferase inhibitor, were added to K-H buffer. The K-H buffer was kept at 37 °C and equilibrated with 95% O₂ and 5% CO₂ throughout the experiment. The hearts were subjected to I/R following a stabiliza-

tion period of 30 min. Normothermic ischemia was induced by perfusing the hearts for 30 min with glucose-free K-H buffer equilibrated with 95% N₂ and 5% CO₂. Reperfusion was carried out by perfusing the hearts for 30 min with K-H buffer equilibrated with 95% O₂ and 5% CO₂ following the ischemic period. The coronary effluent was collected in 10-min intervals. Ten milliliters of effluent collected in this manner was ultrafiltrated at 10000 M.W. using an Amicon Ultra centrifugal filter device (Millipore, U.S.A.) for sample concentration. The precipitate was resolved in 200 μ l of lysis buffer (0.1 M Tris–HCl (pH 6.5), 1 M NaCl, and 1 M glycerol).

Protease Determination The measurement of protease activity was carried out according to the modified method of McEuen *et al.*⁵⁾ Briefly, tryptase-like activity in the effluent was monitored by the hydrolysis of 50 μ M L-Pyr-Gly-Arg-MCA (Peptide Institute, Inc., Osaka) in 1 M glycerol, 0.1 M Tris–HCl (pH 8.0) containing 1% BSA, and 100 μ g/ml heparin. Assays were performed using microtitre plates (total reaction volume=200 μ l). The rate of change in the fluorescence intensity of released 7-amino-4-methyl-coumarin (AMC) (ex: 360 nm em: 465 nm) was measured over 24 h at 37 °C. This rate was confirmed by a preliminary experiment demonstrating that the increase in fluorescence intensity was linear for up to 24 h. The optimal unit of enzymatic activity was expressed by the increase in the ratio of μ mol/l AMC per hour, using the standard AMC curve.

Biochemical Determinations Histamine contents were determined with a commercial kit (Immunotech: A Beckman Coulter Co., France). CPK contents were determined with a commercial kit (Wako pure chemical industries, Ltd., Osaka Japan).

Statistical Analysis The results are expressed as the mean $\pm$ S.E.M. Statistical comparison was performed by unpaired Student's *t*-test. *p* values of less than 0.05 were considered to be significant.

RESULTS

Tryptase-like protease was rapidly released from the heart during ischemia, *i.e.*, within 10 min. The level was gradually

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decreased during ischemia. After reperfusion, the protease level decreased to a level lower than the level of stabilization (Fig. 1A). Similarly, histamine was rapidly released from the heart during ischemia, *i.e.*, within 10 min; following reperfusion, the histamine levels decreased to the level of stabilization (Fig. 1B). However, CPK leakage gradually increased from 20 min after ischemia; following reperfusion, CPK leakage also increased (Fig. 1C).

The trypsin-like protease activity in the effluent was inactivated by leupeptin (Peptide Institute, Inc., Osaka, Japan), the inhibitor of serine and cysteine proteases, in a concentration-dependent manner (Fig. 2). Table 1 summarizes the inhibition of trypsin-like activity by various protease inhibitors in the effluent. The coronary effluents were pre-incubated with the protease inhibitors at 0 °C for 60 min before the reaction with the substrate was carried out. The following inhibitors were used: leupeptin, phenylmethylsulfonyl fluoride (PMSF: SIGMA, U.S.A.: inhibitors of serine protease); E-64 (Peptide Institute, Inc., Osaka: an inhibitor of cysteine pro-

teases); pepstatin A (Peptide Institute, Inc., Osaka: an inhibitor of aspartic proteases); and chymostatin (Peptide Institute, Inc., Osaka: a selective inhibitor of chymotrypsin-like serine proteases). The pattern of inhibition was similar to those of guinea pig and human lung trypsin, as reported by McEuen *et al.*⁵⁾

DISCUSSION

In this study, we determined the time-course of trypsin release from the I/R guinea pig heart. To detect trypsin release from the heart, we measured the enzymatic activity in the coronary effluent. At present, human trypsin can be detected by immunological methods using monoclonal antibody. However, guinea pig trypsin cannot yet be measured by immunological approaches. As regards the characterization of guinea pig trypsin, only the molecular mass, substrate specificity, and inhibitor specificity of partially purified guinea pig lung trypsin has been reported by McEuen *et al.*⁵⁾ Therefore, we measured enzymatic activity according to their method in order to detect trypsin release from the isolated guinea pig heart. McEuen *et al.* recommended Pyr-Pro-Arg-pNA and Pyl-Gly-Arg-pNA as specific substrates for guinea pig trypsin. The activity associated with the human trypsin substrate Bz-DL-Arg-pNA is well known, but the molar catalytic activity associated with Bz-DL-Arg-pNA in the case of guinea pig lung trypsin is very low.⁵⁾ The trypsin-like protease released from the ischemic guinea pig heart had similar inhibition characteristics to those of guinea pig and human lung trypsin (Table 1). In this study, a trypsin-like enzyme was released into the coronary effluent during ischemia, but not during reperfusion. Furthermore, since the time-course of histamine release was similar to that of the trypsin-like protease, it is possible that the source of the protease was mast cells. In addition, CPK leakage was not corresponding to the

Fig. 1. Time-Course of Trypsin-Like Enzyme (A), Histamine (B) and CPK (C) Release from Guinea Pig Hearts by I/R

Data are expressed as the mean $\pm$ S.E.M. ($n=5$). ** $p<0.01$, * $p<0.05$ compared with 0 min.

Fig. 2. Effect of Leupeptin on Trypsin-Like Activity in the Effluent from Guinea Pig Hearts during Ischemia

Data are expressed as the mean $\pm$ S.E.M. ($n=3$). ** $p<0.01$, * $p<0.05$ compared with 0 mM.

Table 1. Effects of Protease Inhibitors on Trypsin-Like Activity in the Effluent from Guinea Pig Hearts during Ischemia

Substrate sample inhibitor	Pyr-Gly-Arg-MCA Effluent (10 min)		Pyr-Pro-Arg-pNA		Human lung trypsin ⁵⁾	
	Conc.	% inhibition	Guinea pig lung trypsin ⁵⁾ Conc.	% inhibition	Conc.	% inhibition
PMSF	1 mM	-2	1 mM	44	1 mM	2
Leupeptin	1 mM	98	0.5 mM	97	0.5 mM	97
E-64	1 mM	6	5 μ M	3	5 μ M	4
Pepstatin A	1 mM	-42	1 μ M	1	1 μ M	9
Chymostatin	1 mM	46	0.5 mM	23	0.5 mM	16

Data shown in italics is from McEuen *et al.*⁵⁾

tryptase and histamine releases. CPK leakage was observed from 20 min after ischemia. It is suggested that tryptase release may not result from cell injury. Therefore, we estimated that the released tryptase-like protease was guinea pig mast cell tryptase. In clinical studies, Filipiak *et al.* reported that the higher serum tryptase levels were observed in patients after myocardial infarction.⁶⁾ In contrast, van Haelst *et al.* reported that the serum levels of tryptase are not elevated in patients with acute coronary syndromes.⁷⁾ In these cases, since the amount of tryptase released by ischemia from cardiac mast cells was low, there is a possibility that it was diluted in the whole blood.

The released tryptase may play either a protective or injurious role on heart I/R injury. Mast cell tryptase is able to activate PAR-2, a protease-activated receptor subtype.⁸⁾ Napoli *et al.* have shown that myocardial function and release of creatine kinase after reperfusion recovered by treatment of PAR-2 activated peptide and exogenous trypsin at ischemia in isolated rat heart transient I/R model (20 min ischemia and 60 min reperfusion).⁹⁾ Furthermore, it has been reported that PAR-2-dependent signaling events are involved in heart protection by ischemic-preconditioning.¹⁰⁾ Since the highest levels of tryptase release were observed within 10 min of the onset of ischemia in this study, it is possible that tryptase is released during ischemic preconditioning usually caused by 5 min ischemia. Therefore, the activation of PAR-2 through tryptase release from heart mast cells possibly involve some of the protective mechanisms of ischemic preconditioning. On the other hand, it is known that proteases such as tryptase have a deleterious effect in the cardiovascular system. Tryptase and chymase released from mast cells are participated in the inflammation and the tissue remodeling through activation of matrix metalloproteinases, apoptosis of cardiac myocytes and proliferation of fibroblasts.¹¹⁾ These actions of tryptase maybe involves in the pathogenesis of myocardial ischemia. Therefore, it is possible that tryptase, released dur-

ing ischemia, deteriorates prognosis of ischemic heart disease on the long-term. Moreover, it has been reported that protease inhibitor leupeptin attenuates myocardial stunning resulting from ischemia in isolated rat heart.¹²⁾

In conclusion, the present study provided experimental evidence that tryptase is released due to transient ischemia in the guinea pig heart. Further investigation is required to elucidate the role of mast cell tryptase in I/R heart injury.

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