Effects of Pharmacological Suppression of Plasminogen Activator Inhibitor-1 in Myocardial Remodeling After Ischemia Reperfusion Injury

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Summary

Plasminogen activator inhibitor-1 (PAI-1) contributes to cardiac ventricular remodeling because migration of inflammatory cells and attenuation of extracellular matrix degradation are caused by plasmin and matrix metalloproteinase. However, the roles of PAI-1 in myocardial ischemia reperfusion (I/R) injury and the following inflammatory response have not yet been well elucidated. To clarify the role of PAI-1 in myocardial I/R injury, we used a specific PAI-1 inhibitor (IMD-1622) in a rat model. The left anterior descending coronary artery was ligated and reperfusion was performed by loosening the suture after 30 minutes of arterial occlusion. A single administration of IMD-1622 (20 mg/kg) or vehicle was given intraperitoneally and then the rats were sacrificed on day 1 or day 14 after I/R. Blood pressure, echocardiograms, histopathology, and molecular examination were performed. The examinations revealed that PAI-1 inhibitor showed limited effects on cardiac dysfunction and ventricular remodeling after I/R. We conclude that the pharmacological inhibition of PAI-1 may not affect ventricular remodeling after myocardial I/R injury. (Int Heart J 2011; 52: 388-392)

Key words: Plasminogen activator inhibitor-1, Chemical compound, Myocardial remodeling, Ischemia reperfusion, Fibrosis

Ischemia/reperfusion (I/R) is a common antecedent event that predisposes a patient to congestive heart failure (CHF). Loss of cardiac function following I/R occurs in the context of myocyte death and interstitial fibrosis, and this is referred to as ventricular remodeling. Recent studies demonstrated that inflammatory responses may cause myocardial damage and fibrosis, leading to progressive impairment of cardiac function. Specifically, matrix metalloproteinases (MMPs), cytokines and chemokines are important mediators in I/R pathogenesis. This possibly represents a vicious cycle operative in the development of myocardial remodeling. Considering the harmful infiltration of macrophages, suppression of the inflammatory factors is effective to attenuate left ventricular (LV) remodeling after I/R.

Plasminogen activator inhibitor (PAI)-1 belongs to a superfamily of serine protease inhibitors and it is normally only expressed in adipocytes and endothelial cells. Previous studies showed that PAI-1 has a key role in thrombosis by way of inhibiting plasminogen activators (PAs) and this results in decreased plasmin activity. Furthermore, PAI-1 is considered to be a new cardiovascular risk factor. It was reported that plasma PAI-1 levels were significantly higher in patients with ST elevation myocardial infarction (STEMI) compared to non-STEMI. Meltzer, et al also revealed that PAI-1 levels appear to reflect cardiovascular risk factors. Therefore, inhibiting PAI-1 activity may decrease clinical cardiovascular risk.

Recently, we developed a specific PAI-1 inhibitor (IMD-1622; 3-(3,4-Dichloro-benzyl)-5-(3,4,5-trihydroxy-benzylidene)thiazolidine-2,4-dione). The in vitro and in vivo inhibitory effects of IMD-1622 were confirmed using a tPA assay, SDS-PAGE, and a rat aorta-vein shunt model. We also reported the effects of IMD-1622 on arterial remodeling, myocarditis, and cardiac transplantation. However, no study has examined the effect on myocardial I/R injury. Thus, the objective of this study was to clarify the role of PAI-1 and the effect of its inhibition on ventricular remodeling after myocardial I/R injury.

Methods

Rat myocardial ischemia/reperfusion injury models: Eight to 10-week-old male Sprague-Dawley rats (6 weeks old, 200 to 250 g) were purchased from Sankyo Laboratories (Tokyo). Rats were anesthetized with 40 mg/kg sodium pentobarbital.
intraperitoneally (i.p.) immediately before operation. Rats were intubated orally with a polyethylene tube for artificial respiration (SN-480-7, Shinnano, Tokyo). The left anterior descending coronary artery was visualized using microscopy and ligated with a 6-0 silk suture. Myocardial ischemia was confirmed by epicardial cyanosis and wall asynergy. After 30 minutes of coronary artery occlusion, reperfusion was performed by loosening the suture and then verified visually. The chest wall and the skin were then closed with a 3-0 silk suture. 

This investigation conforms with the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Treatment protocols: PAI-1 inhibitor (IMD-1622) was kindly provided by the Institute of Medicinal Molecular Design (Tokyo); it was administered at 20 mg/kg per shot i.p. 

The rats were assigned randomly into 3 groups as follows: (i) daily IMD-1622 (20 mg/kg, i.p.) administration that was started during LAD ligation (day 0) and sacrifice on day 1 (n = 7) or day 14 (n = 6); (ii) daily vehicle administration started during LAD ligation (day 0) and sacrifice on day 1 (n = 7) or day 14 (n = 6); and (iii) sham operated rats with daily IMD-1622 (20 mg/kg, i.p.) administration and sacrifice on day 1 (n = 7) or day 14 (n = 6).

Hemodynamic measurements: Blood pressure and heart rate of all rats were evaluated on days 0, 1, 7, and 14. Blood pressure (systolic, diastolic, and mean pressure) was measured in conscious rats using a tail-cuff system (BP-98A, Softron Co., Tokyo).

Echocardiogram: Rats were mildly anesthetized with sodium pentobarbital (40 mg/kg). Transthoracic echocardiography was performed with a commercially available ultrasound (Nemio, Toshiba, Tokyo) before the operation, and 1, 7 and 14 days after reperfusion. A 7-MHz annular array transducer was used. Hearts were imaged in the two-dimensional mode in short-axis views at the level of papillary muscle. M-mode views were used to measure the LV dimensions according to the American Society for Echocardiography leading edge method. 

LV end-diastolic dimension (LVDd) and end-systolic dimension (LVDs), and fractional shortening (%FS = [(LVDd−LVDs) / LVDd]×100) were calculated from the M-mode recordings. 

Measurement of area at risk and infarct size: On day 1 after reperfusion, the anesthetized rats were intubated and thoracotomy was repeated. The left anterior descending artery was ligated tightly and Evans blue dye (1 mL of 2.0% solution) was infused via the inferior vena cava to determine the nonischemic zone (area at risk, AAR) as described earlier. After 30 minutes of coronary artery occlusion, reperfusion was performed by loosening the suture and sacrifice on day 1 (n = 7) or day 14 (n = 6).

Histopathology: To examine the reperfused myocardium pathologically, the hearts were cut at the level of the papillary muscles and harvested in 10% formalin solution. We obtained 4 transverse sections per heart for histopathologic examination. 

Apex, midventricular, and basal level slices were stained with hematoxylin and eosin (HE) and Mallory. The area of myocardium and surrounding tissue affected by I/R (consisting of inflammatory cells, myocardial necrosis, and fibroblasts) was determined with a computer-assisted analyzer (Scion Image beta 4.0.2) in the Mallory staining. The area ratio (affected/LV as a percentage) was calculated as described previously. 

Values for 3 ventricular regions were averaged for each heart, and the mean percentage of affected area for each group was calculated. All data were analyzed in a blind fashion by two independent investigators and averaged.

Immunohistochemistry: To perform immunohistochemistry of reperfused myocardium, rat hearts were cut at the level of the papillary muscles and frozen in O.C.T. compound (Sakura Finetek, Tokyo). Each section (5 μm) was then incubated with anti-rat CD4 (OX-35), CD8 (OX-8), CD11b (WT.5) (PharMinGen, San Diego, CA), MMP-2, MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA) or CD68 (ED1, AbD serotec, Oxford, UK) antibody (each at 1 to 10 μg/mL) for 8 hours at 4°C. Histofine simple stain rat MAX-PO (MULTI) was used as a secondary antibody. Incubations with secondary antibodies were carried out at room temperature for 30 minutes. Finally, each section was reacted with 3-aminoo-9-ethylcarbazole (AEC) matrix solution for 5 to 20 minutes. Immunostained type- and class-matched nonimmune PBS was used as the negative controls for each antibody. Immunohistochemical analysis was performed using quantitative scoring; 0 (absent) to 3 (diffuse). 

Cell preparation and real-time RT-PCR: Cardiac fibroblasts from 4-day-old Sprague-Dawley rats were isolated, subjected to Percoll gradient centrifugation, and cultured in vitro. The cardiac fibroblasts were incubated in DMEM (Sigma-Aldrich Japan, Tokyo) supplemented with 10% FBS for 24 hours at 37°C and starved with 0.25% FBS for 24 hours. Cardiac fibroblasts were treated with or without IMD-1622 (100 nM) and then cultured with or without TNF-α (10 ng/mL) (Peprotech Inc., Rocky Hill, NJ) for 24 hours. Total RNA was isolated from cultured cells using the TRIsure (Bioline Inc., London, UK) and cDNA was prepared with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Japan, Tokyo). Real-time PCR in a StepOne real-time PCR system (Applied Biosystems) was used to determine the mRNA expression of monocyte chemoattractant protein (MCP)-1 (Assay ID: Rn00580555_m1) and MMP-9 (Assay ID: Rn0079162_m1) and 18s rRNA (Assay ID: Hs99999901_s1) as a control. 

Statistical analysis: Values are presented as the mean ± SEM. Groups were compared with ANOVA (Stat View, SAS Institute, Inc.) followed by Scheffé’s test. Student’s t-test was used to compare 2 groups in the second experiment. Differences were considered statistically significant at a value of P < 0.05.

**Results**

PAI-1 inhibitor did not affect blood pressure and heart rate: There was no significant difference in heart rate or mean blood
pressure between the PAI-1 inhibitor-treated and vehicle-treated groups (Figure 1).

**PAI-1 inhibitor did not change myocardial function after reperfusion:** M-mode echocardiograms showed that severely impaired LV anterior wall contraction and thinning was observed in the vehicle-treated group, while good contraction and no impaired wall movement was observed in normal rat hearts. The PAI-1 inhibitor groups also attenuated the regional wall motion 14 days after reperfusion (n = 6, each). There was no significant difference in FS between the PAI-1 inhibitor and vehicle groups (Figure 2).

**PAI-1 inhibitor did not affect infarct size:** No significant difference was observed in the infarction/AAR ratio between the vehicle and PAI-1 inhibitor-treated groups one day after reperfusion (Figure 3).

**PAI-1 inhibitor did not prevent myocardial fibrosis after reperfusion:** Photomicrographs of LV sections with Mallory staining showed an increased fibrotic cross-sectional area in I/R, and PAI-1 inhibitor did not significantly attenuate fibrosis compared to the vehicle. One day after reperfusion, no fibrosis was observed either in the PAI-1 inhibitor-treated hearts or in the vehicle-treated hearts (n = 7). On day 14 after reperfusion, the PAI-1 inhibitor-treated groups showed the same amount of interstitial fibrosis as the vehicle-treated group (n = 6) (Figure 4).

**PAI-1 inhibitor did not alter inflammatory cell migration:** Immunohistochemistry demonstrated increased expression of

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**Figure 1.** PAI-1 inhibitor did not affect blood pressure and heart rate. There was no significant difference in heart rates (A) and mean blood pressure (B) between the PAI-1 inhibitor-treated groups and the vehicle-treated group.

**Figure 2.** PAI-1 inhibitor did not change myocardial function after reperfusion. M-mode echocardiogram showed that severely impaired LV anterior wall contraction was observed in the vehicle-treated group (B), while good contraction and no impaired wall movement was observed in normal rat hearts (A). The PAI-1 inhibitor groups also attenuated the regional wall motion 14 days after reperfusion (C). There was no significant difference in relative FS between the PAI-1 inhibitor and the vehicle groups (D).

**Figure 3.** PAI-1 inhibitor did not affect infarct size. No significant difference was observed in the infarction/AAR ratio between the vehicle (A) and the PAI-1 inhibitor (B) treated groups one day after reperfusion. The graph shows the quantitative results (C).

**Figure 4.** PAI-1 inhibitor did not prevent myocardial fibrosis after reperfusion. Photomicrographs of LV sections with Mallory staining showed an increased fibrotic cross-sectional area in vehicle group (A). The PAI-1 inhibitor (B) did not significantly attenuate fibrosis compared to the vehicle. On day 14 after reperfusion, the PAI-1 inhibitor-treated groups showed the same amount of interstitial fibrosis as the vehicle-treated group (C, n = 6, each).
CD4, CD8, MMP-2, MMP-9, and ED-1 in I/R hearts compared to the native hearts. PAI-1 inhibitor did not significantly alter the expression on day 14 ($n=4$) (Figure 5).

**PAI-1 inhibitor did not alter inflammation-related mRNA levels**: Real-time RT-PCR revealed that MMP-9 and MCP-1 levels were markedly elevated in the vehicle-treated group compared to the native group. PAI-1 inhibitor did not significantly alter the levels ($n=6$) (Figure 6).

**DISCUSSION**

This study showed that pharmacological inhibition of PAI-1 did not contribute to protection against myocardial remodeling after I/R injury in this model. At first, we expected that the PAI-1 inhibitor would suppress myocardial I/R injury, because enhanced PAI-1 expression contributes to cardiac ventricular remodeling through migration of inflammatory cells and attenuation of extracellular matrix degradation. However, no significant differences were observed in blood pressure, ventricular contraction, interstitial fibrosis, or cell infiltration between the vehicle and PAI-1 inhibitor-treated groups.

The correlation between prognosis and the development of cardiac fibrosis is still controversial. Fatal cardiac rupture occurs when fibrinolysis is excessively induced by plasminogen activators. In addition, it has been demonstrated that PAI-1 is related to both inflammatory and anti-inflammatory responses. This complicated behavior of PAI-1 may be caused by an alteration of MMP activity. During the development of cardiac remodeling, fibroblasts are recruited to the injury area and secrete MMPs and chemokines such as MCP-1 involved in cardiac remodeling. Therefore, we investigated the effect on inflammation-related gene expression involved in cardiac remodeling such as MMP-9 and MCP-1 with PAI-1 inhibition in fibroblasts. However, PAI-1 inhibitor did not suppress mRNA expression of these genes.

PAI-1 normally inhibits plasmin and MMP activation via inhibition of plasminogen activators. This is a beneficial side of PAI-1. Thus, decreased activity of plasmin and MMPs prevents cardiac rupture but impairs cardiac function after myocardial infarction. There is also a study indicating that inhibition of u-PA or MMPs prevents cardiac injury and dysfunction. Decreased oxidative inactivation of PAI-1 by myeloperoxidase deficiency contributes to a reduction of LV dilation and preserves LV function. In contrast, after coronary artery occlusion, PAI-1 knockout mice showed a greater infarct area, increased inflammation and enhanced intramyocardial hemorrhage compared to wild type mice. In addition, decreased uPA activity in mice with PAI-1 treatment after viral myocarditis resulted in reduced inflammation and decreased collagen deposition. PAI-1 treatment prevented the resulting cardiac dilation and dysfunction. These investigations imply that PAI-1 has some protective effect on myocardial inflammation. Moreover, PAI-1 has both cell-migration and anti-cell-migration effects. Although this contrary role of PAI-1 still remains unclear, the recent concept of “uPAR-uPA-PAI-1 signaling” may help us understand this complicated situation. uPAR-uPA-PAI-1 signaling is rather complex and not a simple ligand-receptor induced response. The system might involve interference with uPAR-uPA signaling, integrin signaling, and endocytosis related signaling. This means pathways modulated by PAI-1 might include ERK1/2, AKT/PI3K, and the JAK/STAT pathways. In other words, PAI-1 is involved in the proliferation, migration, adhesion, endocytosis, and antiapoptosis of cells. Thus, PAI-1 inhibition is either beneficial or harmful in various situations. Although the PAI-1 inhibitor could act as an anticoagulant or anti-inflammatory agent, our study reveals that pharmacological PAI-1 inhibition does not alter pathological remodeling after myocardial I/R injury. Since we do not fully understand the pathophysiological mechanism, further investigation is needed.
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REFERENCES