Ablation of Angiotensin IV Receptor Attenuates Hypofibrinolysis via PAI-1 Downregulation and Reduces Occlusive Arterial Thrombosis

Yasushi Numaguchi, Masakazu Ishii, Ryuji Kubota, Yasuhiro Morita, Koji Yamamoto, Tadashi Matsushita, Kenji Okumura, Toyoaki Murohara

Objectives—Reduced fibrinolytic activity is associated with adverse cardiovascular events. Although insulin-regulated aminopeptidase (IRAP) was recently identified as the angiotensin (Ang) IV receptor (AT4R), the impact of AngIV-AT4R signaling distal to AngII on the activation of type-1 plasminogen activator inhibitor (PAI-1) in the fibrinolytic process and subsequent formation of thrombosis remains unclarified.

Methods and Results—To determine whether AngIV would inhibit fibrinolysis via PAI-1 activation and promote thrombosis, we evaluated the degree of fibrinolysis in thrombosis models and investigated the roles of AT4R after vascular injury using IRAP knockout mice (IRAP−/−). In endothelial cells from control mice (WT; C57Bl6/J), both AngII and AngIV treatments increased PAI-1 mRNA expression in a dose-dependent manner, whereas the response was blunted in endothelial cells from IRAP−/− mice. FeCl3-induced thrombosis was suppressed in the carotid arteries of IRAP−/− mice when compared with WT mice. Similarly, in a model of carotid artery ligation and cuff placement, IRAP−/− mice demonstrated accelerated fibrinolysis 7 days after surgery and reduced occlusive thrombosis with negative remodeling at 28 days.

Conclusions—AngIV-AT4R signaling has a key role in fibrinolysis and the subsequent formation of arterial thrombosis after vascular injury. AT4R may be a novel therapeutic target against cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2009;29:2102-2108.)

Key Words: AT4R ■ fibrinolysis ■ PAI-1 ■ thrombosis ■ insulin-regulated aminopeptidase

The renin–angiotensin system (RAS) is a key regulator of blood pressure and fluid homeostasis. The main effector peptide of the RAS, angiotensin II (AngII; an octapeptide hormone), causes vasoconstriction, platelet aggregation, and increased sodium uptake and water retention in the kidneys, thereby leading to hypertension, atherosclerosis, and subsequent cardiovascular disease.1 AngII exerts these actions through the activation of downstream signals by binding to its receptors, namely, angiotensin type 1 and type 2 receptors (AT1R and AT2R, respectively). Most of the well-known functions of AngII in the cardiovascular system are mediated through AT1R.2–5 In addition to AngII and these receptors, AngIV, a hexapeptide that is derived by the cleavage of 2 N-terminal amino acids from AngII by aminopeptidases, is sufficiently bioactive to have a prothrombotic potential through the production of plasminogen activator inhibitors (PAIs) by binding to the AT4 receptor (AT4R).6,7 However, its role in the pathogenesis of thrombosis remains unknown.

Insulin-regulated aminopeptidase (IRAP), a zinc-metallopeptidase, was identified as AT4R.8 IRAP was first identified as a major protein in intracellular vesicles isolated from low-density microsomes of rat fat and muscle cells that also colocalized with the insulin-responsive glucose transporter isotype GLUT4.9,10 In 1995, we purified placental leucine aminopeptidase (P-LAP, EC:3.4.11.3, 1024 amino acids, 170 kDa) from retroplacental serum and cloned P-LAP from a human placental cDNA library.11 It was identified as a human homologue of IRAP and confirmed to be a key enzyme regulating the serum levels of hormones such as oxytocin and vasopressin, thus maintaining homeostasis during pregnancy and tumorigenesis.12,13

Impaired fibrinolysis has been linked to thrombosis in a number of experimental and clinical studies.14,15 During fibrinolysis, type-1 PAI (PAI-1; 379 aa and 48 000 MW) binds to tissue-type and urinary-type plasminogen activators (t-PA and u-PA, respectively), protecting a blood clot from premature lysis.16–18 Under atherosclerotic conditions, PAI-1 is overexpressed in vessel walls and plaques, negatively regulating thrombolysis, and this may facilitate thrombotic events after the rupture of plaques.19 Although AngIV and

Received September 2, 2008; revision accepted September 2, 2009.

From the Departments of Medical Science of Proteases (Y.N., M.I.), Cardiology (Y.N., M.I., R.K., Y.M., K.O., T. Murohara), Oncology and Hematology (T. Matsushita), and Cardiovascular Medicine (K.O.), Nagoya University School of Medicine, Japan; and the Department of Transfusion Medicine (K.Y.), Nagoya University Hospital, Japan.

Correspondence to Yasushi Numaguchi, MD, PhD, Department of Cardiology, Nagoya University Graduate School of Medicine, and Department of Medical Science of Proteases, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan. E-mail numa2@med.nagoya-u.ac.jp

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.195057
AT4R are involved in the regulation of thrombus formation and inflammation under thrombogenic conditions such as atherosclerosis, acute myocardial infarction, and pregnancy, little is known as to how the AnglV/AT4R pathway could affect thrombosis, inflammation, and subsequent vascular lesion formation through induction of PAI-1. In this study, we evaluated both acute fibrinolysis and chronic thrombosis using 3 different mouse models: the acute disseminated intravascular coagulation (DIC) model induced by endotoxinemia, the ferric chloride (FeCl3)-induced thrombosis model, and the ligation and cuff placement model in the carotid artery. We aimed to determine whether the genetic ablation of AT4R could attenuate thrombosis and inflammation using IRAP-deficient mice (IRAP<sup>−/−</sup>). Our data provide an important insight into the novel association between RAS and thrombosis and heightens awareness to a pivotal role of IRAP/AT4R in the pathogenesis of cardiovascular disease.

**Methods**

**Animals**

The derivation of IRAP<sup>−/−</sup> mice has been described in detail elsewhere. The mice (a kind gift from Bayer AG, Leverkusen, Germany) were derived from a 129/C57BL/6 background, and backcrossed with a C57BL/6 strain more than 7 times. C57BL/6 mice were used as wild-type (WT) controls. Animal experiments were performed with the authorization of the institutional review board of the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. This animal study conformed to the “Position of the American Heart Association on Research Animal Use” (Circulation 1985; 71:849A). All experiments were performed using 8- to 10-week-old male mice, unless otherwise indicated.

**Cell Culture and Drug Treatment**

Murine aortic endothelial cells (ECs) were explanted from the mouse aorta as previously described. ECs of passages 2 and 3, which were confirmed by CD31-positive staining, were used in the experiments (please see supplemental Figure I, available online at http://atvb.ahajournals.org).

**Measurement of mRNA Expression Level**

Total RNA was extracted from cell lysates using TRIzol Reagent (Invitrogen), and the amount was quantified by a densitometer. The first cDNA strand was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using the LightCycler<sup>®</sup> System (Roche Diagnostics) and QuantItect SYBR Green PCR kit (Qiagen; please see supplemental materials).

**Hemodynamic Analyses and Tail Bleeding Times**

Systolic blood pressure and heart rate of mice (n=12, each) were determined by the tail-cuff detection system Softron BP-98A (Softron). Tail bleeding times were assessed by standard procedures at 8 weeks of age (n=6, each; please see supplemental materials).

**Hemostatic Analyses**

Hematologic Analyses

The mice (n=8 to 10, each) were anesthetized by sodium pentobarbital (Dainippon Pharmaceutical, Tokyo, Japan, 33 mg/kg IP), and blood was drawn with 21G needles by the ventricle puncture method via the subxyphoid approach. Differential blood counts were obtained using an automated hematology analyzer. Samples of mouse plasma after LPS injection were stored at −80°C before measurement. To detect plasma proteins such as D-dimer and thrombin-antithrombin III complex, ELISA kits were used (please see supplemental materials).

To assess the fibrinolytic balance between PAI-1 and t-PA, we evaluated the active antigen levels of PAI-1 and t-PA using murine PAI-1 and t-PA activity assay kits, respectively (Innovative Research Inc).

**LPS Treatment for the DIC Model**

Eighteen to 20-week-old mice (n=12, each) were treated intraperitoneally with 100 µL of LPS (50 mg/kg) and euthanized at 2, 4, 8, and 24 hours after injection. We assessed the levels of active PAI-1 in platelet-poor plasma (PPP) and the degree of fibrin deposition in the kidney.

**FeCl<sub>3</sub>-Induced Arterial Injury and Thrombosis**

Mice were subjected to carotid artery injury with 10% FeCl<sub>3</sub> (n=15 to 20 in each group). Briefly, mice were anesthetized with sodium pentobarbital, the left carotid artery was dissected, and a small strip of filter paper soaked in 10% FeCl<sub>3</sub> was applied to the surface of the adventitia for 3 minutes. Carotid flow was monitored with a flow probe (0.5VB, Transonic Systems) interfaced with a flowmeter (Power Laboratory, AD Instruments) and analytic program (Laboratory Chart 7, AD Instruments). The time to occlusion was measured.

To assess the effects of PAI-1 inhibition on thrombosis, we administered a specific PAI-1 inhibitor (T-686; a kind gift from Mitsubishi Tanabe Pharmaceutical) by gavage (100 mg/kg/d) for 7 days before the experiments.

**Carotid Artery Ligation and Cuff Placement Procedure and Morphometric Analysis**

To assess the process of thrombolysis, we performed ligation and cuff placement in the carotid artery, which is characterized by blood flow cessation and intracuff EC injury. At 0, 4, 7, and 28 days after surgery, the carotid arteries were removed and stained by hematoxylin and eosin (H&E), Masson trichrome, or Elastica van Gieson staining, followed by morphometric analyses (Please see supplemental materials). T-866 was administered for 7 days before and 28 days after the surgery.

To assess NFκB activation and the inflammatory response, the sections taken 4 days after surgery were immunostained with phosphorylated IκB-α (Abcam, Cambridge) and MCP-1 (rat monoclonal antibody for CCL2, ABR/Thermo Fischer Scientific), visualized with Alexa Fluor 488 (Molecular Probe/Invitrogen), counterstained with DAPI (Molecular Probe), and observed with an epifluorescent microscope (BZ8000, Keyence).

**Statistical Analysis**

The data are presented as mean±SEM values. Statistical analysis of multiple comparisons among the groups was conducted by 1-way ANOVA followed by the Bonferroni test. Statistical analysis of comparisons between 2 groups over time used repeated measures ANOVA. Multiple comparisons in nonparametric analysis were performed by the Kruskal–Wallis test. A probability value of P<0.05 was considered significant.

**Results**

**Hemodynamics and Hemostasis in IRAP<sup>−/−</sup> Mice**

We initially measured the parameters of hemodynamics and hemostasis to determine the basal characteristics of IRAP<sup>−/−</sup>.
mice. Blood pressure, body weight, fat weight evaluated by the ratio of epididymal fat weight to body weight, and heart rate were similar between groups. With respect to glucose and lipid metabolism, IRAP−/− mice showed no differences in the basal levels of glucose, insulin, cholesterol, triglycerides, or free fatty acids, compared with WT. IRAP−/− mice showed no differences in basal hematologic characteristics (please see supplemental Table I).24

**PAI-1 mRNA Expression and Active PAI-1 Levels After LPS Treatment in Cultured ECs**

To assess the roles of AT4R on PAI-1 expression in ECs and subsequent fibrinolysis, we compared the magnitude of PAI-1 mRNA expression between 2 groups in response to LPS treatment, which is a potent activator that induces PAI-1 in ECs to a maximum level. There were no differences observed in PAI-1 mRNA expression between the groups before treatment. After LPS treatment, in ECs isolated from WT and IRAP−/− mice, PAI-1 mRNA levels increased by 4.2±0.5 and 3.8±0.4 fold, respectively (P<0.005 versus control). The active PAI-1 levels in the culture medium after LPS treatment were lower in IRAP−/− mice than in WT mice (57±2.4 and 86±3.5 ng/mL, P<0.01, respectively).

**PAI-1 mRNA Expression After AngII and AngIV Treatment in Cultured ECs**

In ECs from WT mice, PAI-1 mRNA expression increased in a dose-dependent manner and reached a 3.3±0.6 fold increase with 10−6 mol/L AngII (P<0.01 versus control) and a 2.8±0.6-fold increase by 10−6 mol/L AngIV (P<0.01 versus control, Figure 1A). In ECs from IRAP−/− mice, PAI-1 mRNA induction was suppressed after AngII and AngIV treatment (2.0±0.3 and 1.7±0.3-fold at 10−6 mol/L, respectively, P<0.05 versus WT, Figure 1B).

**Active PAI-1 and t-PA Levels After LPS Treatment**

To evaluate the real-time fibrinolytic balance after LPS injection, we observed the alteration of active PAI-1 and t-PA antigen levels in PPP over time. In WT mice, the curve of the active PAI-1 antigen levels was biphasic with peaks 2 and 8 hours after LPS treatment (Figure 2A). In contrast, the active PAI-1 antigen levels had only one peak 2 hours after LPS treatment in IRAP−/− mice, which then gradually decreased. The increases in the active PAI-1 antigen levels 2, 4, and 8 hours after LPS injection in IRAP−/− mice were significantly

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**Figure 1. PAI-1 mRNA expression in ECs.** Alterations of PAI-1 mRNA expression in response to treatments with LPS and serial concentrations of AngII and AngIV. A, In WT mice, PAI-1 mRNA levels increased in a dose-dependent manner. B, In IRAP−/− mice, the response to AngII and AngIV were blunted compared with WT, and the dose-dependency observed in WT mice disappeared. *P<0.05 and †P<0.01 vs control. Results are expressed as mean±SEM n=3 in each group.

**Figure 2.** Time course of active PAI-1 and t-PA antigen levels in plasma after LPS injection. A, In WT mice, the expression pattern was biphasic with peaks 2 and 8 hour after LPS treatment, whereas in IRAP−/− mice, the increase was inhibited and only one peak was observed at 2 hours. Results are expressed as mean±SEM, n=12 in each group. *P<0.05 and †P<0.01 vs WT. B, No differences were observed in t-PA levels between the 2 groups.
lower than those in WT mice ($P<0.01$, $P<0.05$, and $P<0.01$ for $\text{IRAP}^{-/-}$ mice versus WT mice, with respect to each time point). In general, the active PAI-1 antigen levels were suppressed after LPS injection in $\text{IRAP}^{-/-}$ mice compared with those in WT mice ($P<0.001$ each versus WT mice). In contrast to the alteration of active PAI-1 levels, active t-PA levels gradually increased with a peak at 8 hours after LPS injection and no difference was observed between the 2 groups (Figure 2B).

Renal Glomerular Fibrin Deposition After LPS Treatment

As well as acute PAI-1 release from ECs, LPS injection causes a loss of integrity in the EC monolayer of blood vessels. Permeability increases and platelets can adhere to the extracellular matrices (ECMs) beneath the EC layer and activate aggregation, thereby forming fibrin deposits mainly in capillaries and in renal glomeruli. We counted the rate of renal fibrin deposition 4 hours after LPS injection at the point when fibrin deposition is most significant, as observed previously. As shown in Figure 3, the percentage of fibrin deposition observed within glomeruli per section were $12.5\pm3.5\%$ in WT, $3.2\%$ and $14.2\%$ in T-686–treated mice ($P<0.001$ versus WT), respectively. At 28 days, in the sections from WT mice, thrombus formation was confirmed in over 90% of the sections from WT mice 7 days after surgery, whereas in T-686–treated mice and $\text{IRAP}^{-/-}$ mice, fibrinolysis was accelerated and the total area of thrombi in the inner lumen area was as low as $12.5\%\pm3.2\%$ and $14.2\%\pm3.5\%$ ($P<0.001$ versus WT), respectively. At 28 days, in the sections from WT mice, thrombus

Assessment of Occlusive Thrombosis in the Model of Ligation and Cuff Placement

For the assessment of chronic occlusive thrombus formation and fibrinolysis in the artery, we used a newly developed model by combined treatments of murine carotid artery ligation with perivascular cuff placement (supplemental Figure II). We confirmed that the intraluminal thrombus formation was evident 7 days after surgery in WT mice (Figure 5). Thrombi were remarkably apparent 4 days after surgery in sections from WT mice and the percentage was $65.2\%\pm5.0\%$ (Figure 5A). In contrast, the thrombus area was as low as $21.4\%\pm3.1\%$ in $\text{IRAP}^{-/-}$ mice and $24.5\%\pm3.7\%$ in T-686–treated mice ($P<0.01$ versus WT). Organized thrombus formation was confirmed in over 90% of the sections from WT mice 7 days after surgery, whereas in T-686–treated mice and $\text{IRAP}^{-/-}$ mice, fibrinolysis was accelerated and the total area of thrombi in the inner lumen area was as low as $12.5\%\pm3.2\%$ and $14.2\%\pm3.5\%$ ($P<0.001$ versus WT), respectively. At 28 days, in the sections from WT mice, thrombus

![Figure 3. Fibrin deposition in renal glomeruli after LPS injection. Histological analyses of the kidneys from WT and $\text{IRAP}^{-/-}$ mice. The glomerular fibrin deposition was immunostained with a specific antibody for fibrin/fibrinogen (red; B and E). Right panels (C and F) show merged images counterstained with DAPI. The circles and arrows indicate glomeruli and fibrin deposition, respectively. Bars are 50 $\mu$m.](image-url)

![Figure 4. FeCl3-induced arterial thrombosis. A, In WT mice, FeCl3 caused occlusion of the carotid artery in all samples. In T-686–treated and $\text{IRAP}^{-/-}$ mice, the percentages of occlusion were 87% and 72%, respectively (each $P<0.001$ vs WT mice). The representative flow curve of each group is shown. Bars are 1 minute. B, In occluded arteries, the mean time to occlusion was significantly longer in $\text{IRAP}^{-/-}$ and T-686–treated mice than in WT mice. $P<0.01$ and $P<0.001$ vs WT mice.](image-url)
was rarely observed and the lumen was occupied with rigid ECMs such as collagen and elastin produced by protruded myofibroblasts. In T-686–treated mice, the lumen was patent, whereas neointimal formation was observed and the adventitia was rich with myofibroblasts which was similar to the sections from WT mice. In IRAP−/− mice, the lumen was patent and thrombus and neointimal formation were rarely observed. However, negative remodeling (hypertrophic vessel narrowing) at a remote period after injury was observed. The average lumen diameter was smaller than that of WT mice (1.20±0.2 mm, 1.22±0.2 mm and 1.37±0.3 mm, respectively, P<0.05 each, Figure 5B).

Figure 5. A novel arterial injury model combined with ligation and cuff placement for thrombosis. Representative sections of each group at 0, 4, 7, and 28 days after surgery are shown. Bars are 50 μm. A, Note that although no differences were observed in lumen diameters and the areas of the media and adventitia on day 0, the lumens were occluded with organized thrombi in accordance with hyperplasia of the media and adventitia in the arteries of WT mice 7 days after surgery. Diminished cellular proliferation and accelerated fibrinolysis were observed in the arteries of IRAP−/− mice compared with WT mice. B, At 28 days after surgery, the lumen of WT mice was occluded with protruded myofibroblasts and rich ECMs, whereas the lumen of IRAP−/− mice exhibited hypertrophic vessel narrowing without thrombus occlusion depicting the negative remodeling phenomenon. C, Thrombus area per section. The degree of thrombus occlusion was quantified by the percentage of thrombus area from the inner lumen area at 0, 4, and 7 days after surgery (n=6 to 10, each). At 28 days, the sum of neointima-media area and thrombus area were quantified (n=8 to 10, each). Results are expressed as mean±SEM. ⁎P<0.001 vs WT mice.

Discussion
We have tested the importance of the AngIV-AT4R-PAI-1 axis in fibrinolysis and thrombosis by comparing IRAP−/−.
mice with WT controls. In ECs from \textit{IRAP}^{−/−} mice, PAI-1 mRNA induction was blunted after AngII and AngIV treatment when compared to WT mice. We demonstrated that the genetic ablation of AT4R attenuated thrombosis in acute and chronic thrombosis mouse models.

PAI-1, a member of serine-protease inhibitors (serpins), is the important inhibitor of plasma fibrinolytic activity as well as α-2 plasmin inhibitor and serves as a pseudosubstrate for PAs.\textsuperscript{15–18,28–30} Although PAI-1 induction was observed in ECs from each type of mice in response to either AngII or AngIV, the dose-dependency, which was obvious in ECs from WT mice, disappeared in ECs from \textit{IRAP}^{−/−} mice. The ablation of AT4R reduced the susceptibility to both AngII and AngIV; however, PAI-1 expression was not completely abolished by the lack of the \textit{AT4R} gene. This may suggest an interaction between AT1R and AT4R in PAI-1 expression.\textsuperscript{31}

In contrast, when we examined the active PAI-1 antigen levels in the PPP after LPS treatment, the PAI-1 levels of \textit{IRAP}^{−/−} mice were markedly suppressed compared with those of WT mice. As PAI-1 antigen levels increased, active t-PA levels increased concomitantly by 4.1 fold. However, the difference of increases between mice was not observed. These findings suggest that the difference in the process of clot lysis between mice may be due to the magnitude of PAI-1 induction in response to stimuli and the abundance of the active form of PAI-1. In support of this, the degree of renal glomerular fibrin deposition was also suppressed in \textit{IRAP}^{−/−} mice. In a previous report, PAI-1−deficient mice showed lower active PAI-1 antigen levels in plasma after LPS injection and diminished glomerular fibrin deposition.\textsuperscript{20,30} In \textit{IRAP}^{−/−} mice, PAI-1 induction is impaired, and these results are similar to those of PAI-1−deficient mice.\textsuperscript{32}

In the present study, we used a newly developed model using combined treatments of murine carotid artery ligation and perivascular cuff placement.\textsuperscript{23} This model is characterized by chronic blood flow cessation and EC injury limited within an intracuff lesion, which allowed us to quantitate thrombus formation in arteries. Sections from the intracuff lesion from T-686–treated mice and thrombus formation in arteries. Sections from the intracuff within an intracuff lesion, which allowed us to quantitate

Regarding NFκB activation and PAI-1 induction in the injured arteries, although the administration of T-686 could inhibit thrombus formation in the acute phase, T-686 failed to suppress NFκB activation and inflammatory responses. In contrast, both NFκB activation and inflammation were suppressed in the arteries of \textit{IRAP}^{−/−} mice.\textsuperscript{31} In a remote period, sections from T-686–treated mice demonstrated an intermediate phenotype between those of WT and \textit{IRAP}^{−/−} mice for luminal patency, hyperproliferation of myofibroblasts, over-production of ECMs, and neointimal formation. These findings strongly suggest that the AngIV-AT4R pathway could regulate both thrombus formation through PAI-1 induction and inflammation through NFκB activation and leukocyte infiltration. To support this, it has been demonstrated that endosomal peptide trimming by IRAP is essential for MHC class 1 cross-presentation.\textsuperscript{33} This may suggest that the control of IRAP activity leads to suppression of leukocyte infiltration and inflammatory responses as shown in our thrombosis models. We hypothesized that AT4R mediated mechanisms to explain the in vivo biological effects: (1) AngIV binding causes an accumulation of different bioactive peptides by preventing their degradation by IRAP; (2) AngIV binding to IRAP activates certain intracellular signaling pathways and inflammatory responses as shown in our thrombosis models. Further investigations are required to clarify the pivotal role of IRAP in immune response and inflammation.

In conclusion, we have demonstrated that the genetic ablation of IRAP/AT4R attenuated hypofibrinolysis and inhibited thrombosis after arterial injury. These findings may raise awareness of the importance of the AngIV-AT4R axis in the pathogenesis of cardiovascular diseases, and thus the concept of protection distal to AngII and its receptors may open new avenues for therapeutic intervention in patients with cardiovascular disease.

**Acknowledgments**

The animal study was performed mainly at the Institute for Laboratory Animal Research, Nagoya University.

**Sources of Funding**

This study was partially funded by a grant from the Mitsubishi Pharma Research Foundation (Y.N., M.I., and T. Murohara) and a grant for investigating clinical vascular function from the Kimura Memorial Heart Foundation (Y.N.).

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. 2009;29:2102-2108; originally published online September 10, 2009;
doi: 10.1161/ATVBAHA.109.195057

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary data for Methods

**Measurement of mRNA expression levels**

The reactions were carried out under the following conditions for mouse PAI-1 and GAPDH: one cycle of 95°C for 15 min, followed by 50 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. The following primers were designed using Primer Express Software, version 3.0 (Applied Biosystems Japan, Tokyo, Japan) and custom synthesized by Invitrogen: *PAI-1*, forward 5'-CCCCAAATTTTTGGGGG-3', reverse 5'-CCCCAAAAATTTTTGGGGG-3'; *GAPDH*, forward 5'-ATCACCATCTTCCAGGAGCGA-3', reverse 5'-GAGGGGGCAGAGATGATGAC-3'. Analyses were carried out with LightCycler™ 3.5 software (Roche Diagnostics Japan, Tokyo, Japan). The level of expression of each mRNA was normalized to the expression level of GAPDH. The measurements were repeated three times. [1]

**Tail bleeding times**

Tail bleeding times were assessed by standard procedures at 8 weeks of age (*n*=6, each). Mice were anesthetized using sodium pentobarbital and approximately 3 mm was cut from each tail tip to expose the artery and veins. The excised end was then
immersed in a test tube containing saline solution prewarmed to 37°C. Bleeding was monitored visually and the time taken for bleeding to stop was recorded.

**Analyses of platelet aggregation**

The mice (n=8 to 10, each) were anesthetized by sodium pentobarbital (Dainippon Pharmaceutical, Tokyo, Japan, 33 mg/kg IP) and blood was drawn with 21G needles by the ventricle puncture method via the subxyphoid approach. Differential blood counts were obtained using an automated hematology analyzer. To assess collagen- and ADP-induced platelet aggregation, 0.9 ml of blood samples were collected, mixed with 0.1 ml of 3.8% sodium citrate and incubated at 25°C for 1 h before measurement with a platelet aggregometer of whole blood, which was developed from the screen filtration pressure method (WBA-neo, ISK, Tokyo, Japan). The degree of platelet aggregation of each blood sample was evaluated by the platelet aggregation threshold index (PATI; M), that is, the concentration of agonist required with an inducing pressure rate of 50%.

To assess platelet aggregation using plasma, platelet-rich plasma was measured turbidimetrically using an aggregation analyzer (Hematracer 810, MC Medical, Tokyo, Japan) following calibration with platelet-poor plasma. Aggregation was induced by
either collagen (1 or 20 µg/mL) or ADP (0.5 or 10 µM), and the changes in light transmittance were recorded for 6 minutes.

**Detection of various plasma proteins and coagulation factors**

Active PAI-1 and t-PA antigen in plasma, prepared as before, were measured with an ELISA kit using the binding of PAI-1 or t-PA to mouse recombinant u-PA or PAI-1, respectively (murine PAI and t-PA activity assay kit, Innovative Research Inc., Southfield, MI, USA). The D-dimer ELISA kit and reagents for activated partial thromboplastin time (aPTT) were purchased from Diagnostica Stago Inc. (Parsippany, NJ, USA) and the antibodies for thrombin-antithrombin III complex (TAT) ELISA determination were obtained from Enzyme Research Laboratories (South Bend, IN, USA).

**Immunohistochemistry of renal glomerular fibrin deposition**

Immunohistochemical staining was performed using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA, USA) as previously described. Briefly, the tissue sections (5 mm) were deparaffinized and incubated with 10% normal goat serum for 30 min. The slides were then incubated overnight at 4°C with 0.25 mg/ml of goat anti-mouse
fibrinogen/fibrin antibody (GAM/Fbg/7S; Nordic Immunological Laboratory, Tilburg, Netherlands) for the primary antibody and donkey anti-goat IgG conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR) as the secondary antibody. Control slides were incubated with normal goat IgG instead of primary antibody. The sections were observed under an epifluoromicroscope (BZ-8000, Keyence, Osaka, Japan). The glomeruli were confirmed in the sections stained with hematoxylin and eosin (H&E) and fluorescent agents.

**Carotid artery ligation and cuff placement procedure and morphometric analysis**

Mice were anesthetized using sodium pentobarbital and the right common carotid artery and its bifurcation were exposed after a mid cervical incision. The connective tissue around the artery was carefully removed. The artery was then ligated with a 6-0 nylon ligature (6-0 Ethilon, Ethicon, Inc., Brunswick, NJ) just beneath the bifurcation. The nonocclusive polyethylene cuff (PE-50, length 2 mm, inside and outside diameter 0.580 and 0.965 mm, respectively; Becton Dickinson, Sparks, MD, USA) was placed proximal to the ligated site and tied circumferentially with a nylon ligature.

At 0, 4, 7, and 28 days after surgery, the mice were anesthetized using
sodium pentobarbital and perfused through the left cardiac ventricle with 4% paraformaldehyde in PBS (pH 7.4) under physiological pressure (n=6 to 10, each). The carotid arteries were removed and immersed in fixative for 6 h at 4°C. Each section was stained with H&E, Masson trichrome staining, or Elastica van Gieson staining.

The mid internal sections of each carotid artery were examined morphometrically with a computerized digital image analysis system (NIH Image by Wayne Rasband) in a blinded manner by two independent examiners (K.O. and M.I.).

Reference

Figure Legends for Supplementary Figures

Supplementary Figure I

The cultured ECs explanted from the murine aortae. ECs were explanted from the murine aortae and harvested. To verify the identity of ECs, we performed immunostaining with a specific antibody for CD31 (green) and counterstained with DAPI for nuclei (blue). More than 95% of the cultured cells were positive for CD31 (n=6). No apparent morphological difference was observed between ECs from WT and IRAP Knockout mice. Note that the margins of cells were stained in green, confirming ECs.

Supplementary Figure II

A novel arterial injury model combined with ligation and cuff placement for fibrinolysis. (A and B) Macrographs of the carotid artery exercised from WT mice 7 days after surgery. (A) The cuff was placed proximal to the ligated point at the bifurcation. The parenthesis depicts the cuff (PE-50 tube, length 2 mm). (B) The carotid artery detached the cuff. Red line indicates the internal midpoint.
Table I  

Baseline Characteristics of Mice

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<td><strong>General Characteristics</strong></td>
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<td></td>
</tr>
<tr>
<td>Body weight (BW; g)</td>
<td>25.6±1.8</td>
<td>24.9±1.9</td>
</tr>
<tr>
<td>Epididymal fat weight/BW</td>
<td>0.021±0.002</td>
<td>0.020±0.002</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>98.2±8.6</td>
<td>96.5±8.6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>598±55</td>
<td>589±58</td>
</tr>
<tr>
<td><strong>Glucose and Lipid Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>68.6±4.7</td>
<td>65.9±5.8</td>
</tr>
<tr>
<td>Insulin (ng/dL)</td>
<td>1.22±0.31</td>
<td>1.21±0.29</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>83.8±6.5</td>
<td>80.3±6.3</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>72.1±5.7</td>
<td>75.6±6.8</td>
</tr>
<tr>
<td>Free fatty acid (mEQ/L)</td>
<td>572±54</td>
<td>542±48</td>
</tr>
</tbody>
</table>

(Continues on next page)
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>IRAP⁺⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood analysis</strong></td>
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<tr>
<td>White blood cells (/mL)</td>
<td>5510±75</td>
<td>5660±72</td>
</tr>
<tr>
<td>Red blood cells (x10⁴/mL)</td>
<td>877±46</td>
<td>856±50</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.2±0.59</td>
<td>14.5±0.68</td>
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<tr>
<td>Hematocrit (%)</td>
<td>51.2±1.0</td>
<td>50.5±1.6</td>
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<tr>
<td>Platelets (10⁹/mm³)</td>
<td>86.1±5.6</td>
<td>88.2±8.1</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>42.7±0.81</td>
<td>43.7±0.76</td>
</tr>
<tr>
<td>TAT (mg/mL)</td>
<td>23.0±3.1</td>
<td>27.3±3.5</td>
</tr>
<tr>
<td>D-dimer (ng/mL)</td>
<td>20.3±2.6</td>
<td>23.2±2.6</td>
</tr>
<tr>
<td>Bleeding time (min)</td>
<td>1.05±0.12</td>
<td>1.10±0.12</td>
</tr>
<tr>
<td>Active PAI-1 (ng/mL)</td>
<td>0.121±0.0061</td>
<td>0.125±0.0043</td>
</tr>
</tbody>
</table>

Levels of aPTT, TAT, D-dimer, and active PAI-1 in platelet-poor plasma were measured using ELISA kits. At basal levels, there were no significant differences between the two groups.
Figure I
Figure II