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
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 endotoxemia, 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).

ECs cultured to subconfluency in a 6-well plate were treated with 10 μg/mL lipopolysaccharide (LPS; Escherichia coli serotype O111:B4; Sigma Chemical Co Ltd) to assess the induction levels of PAI-1 mRNA after LPS treatment. Cells were then lysed with lysis O111:B4; Sigma Chemical Co Ltd) to assess the induction levels of PAI-1 mRNA after LPS treatment. Cells were then lysed with lysis buffer containing 1% β-mercaptoethanol after washing twice in PBS. Cell lysates were stored at −80°C before mRNA measurement. We evaluated the active antigen levels of PAI-1 in the culture medium using the murine PAI-1 activity assay kit (Innovative Research Inc).

Similarly, ECs were pretreated with 1 μmol/L captopril (Sigma Chemical) overnight and then exposed to AngII and AngIV (Peptide Institute) at 10<sup>−9</sup> to 10<sup>−6</sup> mol/L for 6 hours.

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

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).

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 with haematoxylin and eosin (H&E), Masson trichrome, or Elastica van Gieson staining, followed by morphometric analyses (Please see supplemental materials). T-686 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 Fisher 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±0.2 fold increase with 10⁻⁸ mol/L AngII (P<0.01 versus control) and a 2.8±0.6-fold increase by 10⁻⁹ 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 10⁻⁸ 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
lower than those in WT mice \((P<0.01, P<0.05, \text{and } P<0.01 \text{ for } \text{IRAP}^{-/-} \text{ mice versus WT mice, with respect to each time point})\). In general, the active PAI-1 antigen levels were suppressed after LPS injection in \(\text{IRAP}^{-/-}\) mice compared with those in WT mice \((P<0.001 \text{ each versus WT mice})\). 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 \(18.9\pm2.5\%\) in WT and \(4.32\pm1.9\%\) in \(\text{IRAP}^{-/-}\) mice \((P<0.01 \text{ versus WT})\).

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 \((P<0.01 \text{ versus WT})\). 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 \(14.2\pm3.5\%\) \((P<0.001 \text{ versus WT})\), respectively. At 28 days, in the sections from WT mice, thrombus...
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).

Immunostaining of sections from the carotid arteries revealed that NFκB activation (confirmed with phosphorylated IκB-α) was prominent in T-686–treated and WT mice, whereas NFκB activation was suppressed in IRAP−/− mice (Figure 6). As NFκB activity was attenuated, the expression levels of inflammation markers such as MCP-1 was concomitantly suppressed in the sections from IRAP−/− 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 IRAP<sup>−/−</sup> 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. 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 IRAP<sup>−/−</sup> 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 AT4R gene. This may suggest an interaction between AT1R and AT4R in PAI-1 expression.

In contrast, when we examined the active PAI-1 antigen levels in the PPP after LPS treatment, the PAI-1 levels of IRAP<sup>−/−</sup> 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 IRAP<sup>−/−</sup> 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. In IRAP<sup>−/−</sup> mice, PAI-1 induction is impaired, and these results are similar to those of PAI-1–deficient mice.

In the present study, we used a newly developed model using combined treatments of murine carotid artery ligation and perivascular cuff placement. 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 PAs. Although PAI-1 induction was observed in WT mice similar to those observed in IRAP<sup>−/−</sup> mice, suggesting that reduced occlusive thrombosis in IRAP<sup>−/−</sup> mice was at least, in part, attributable to suppressed induction of PAI-1.

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 IRAP<sup>−/−</sup> mice. In a remote period, sections from T-686–treated mice demonstrated an intermediate phenotype between those of WT and IRAP<sup>−/−</sup> mice for luminal patency, hyperproliferation of myofibroblasts, overproduction of ECMs, and neo-intimal 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. 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 3 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 (3) AngIV binding to IRAP modulates peptide trimming or proteasomal degradation, thereby regulating the physiological cellular processes. 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**

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**Disclosures**

None.

**References**


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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'-CCCCCAAAATTTTTGGGGG-3', reverse 5'-CCCCCAAAAATTTTTGGGGG-3'; GAPDH, forward 5'-ATCACCATCTTCCAGGACGA-3', reverse 5'-GAGGGGCGAGATGATGAC-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\textsuperscript{-/-} 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>
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<tr>
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<th>WT</th>
<th>IRAP/−</th>
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<tr>
<td><strong>General Characteristics</strong></td>
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<tr>
<td>Body weight (BW; g)</td>
<td>25.6±1.8</td>
<td>24.9±1.9</td>
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<td>Epididymal fat weight/BW</td>
<td>0.021±0.002</td>
<td>0.020±0.002</td>
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<td>Systolic blood pressure (mmHg)</td>
<td>98.2±8.6</td>
<td>96.5±8.6</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>598±55</td>
<td>589±58</td>
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<td><strong>Glucose and Lipid Metabolism</strong></td>
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<tr>
<td>Fasting glucose (mg/dL)</td>
<td>68.6±4.7</td>
<td>65.9±5.8</td>
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<td>Insulin (ng/dL)</td>
<td>1.22±0.31</td>
<td>1.21±0.29</td>
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<td>Cholesterol (mg/dL)</td>
<td>83.8±6.5</td>
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<td>Triglycerides (mg/dL)</td>
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<td>Free fatty acid (mEQ/L)</td>
<td>572±54</td>
<td>542±48</td>
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<tr>
<th></th>
<th>WT</th>
<th>IRAP&lt;sup&gt;+&lt;/sup&gt;</th>
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<tr>
<td><strong>Blood analysis</strong></td>
<td></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 (x10&lt;sup&gt;4&lt;/sup&gt;/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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<td>Hematocrit (%)</td>
<td>51.2±1.0</td>
<td>50.5±1.6</td>
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<tr>
<td>Platelets (10&lt;sup&gt;9&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;)</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>
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<tr>
<td>TAT (mg/mL)</td>
<td>23.0±3.1</td>
<td>27.3±3.5</td>
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<tr>
<td>D-dimer (ng/mL)</td>
<td>20.3±2.6</td>
<td>23.2±2.6</td>
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<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>
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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