Vascular Smooth Muscle Cells Are Responsible for a Prothrombotic Phenotype of Spontaneously Hypertensive Rat Arteries

Karima Ait Aissa,* Jérémy Lagrange,* Amel Mohamadi,* Huguette Louis, Bénédicte Houppert, Pascal Challande, Denis Wahl, Patrick Lacolley, Véronique Regnault

Objective—The hypothesis that hypertension induces a hypercoagulable state arises from the complications associated with hypertension: stroke and myocardial infarction. Here, we determine whether hypertension causes changes in the thrombin-generating capacity of the vessel wall.

Approach and Results—We used spontaneously hypertensive rats (SHR) compared with Wistar rats. The addition of thoracic aortic rings of SHR to a Wistar or SHR plasma pool resulted in a greater increase in thrombin generation compared with equivalent rings from Wistar. This increase occurred in 12- but not 5-week-old rats and was prevented by an angiotensin II–converting enzyme inhibitor, indicating that established hypertension is required to induce increased thrombin generation within the vessel wall. Whereas no difference was observed for endothelial cells, thrombin formation was higher on aortic smooth muscle cells (SMCs) from SHR than on those from Wistar. Exposure of negatively charged phospholipids was higher on SHR than on Wistar rings, as well as on cultured SMCs. Tissue factor activity was higher in SHR SMCs. Twelve-week-old SHR exhibited accelerated FeCl₃-induced thrombus formation in carotid arteries, and the resulting occlusive thrombi were disaggregated by blockade of glycoprotein Ibα–von Willebrand factor interactions. SHR SMCs were more sensitive to thrombin-induced proliferation than Wistar SMCs. This effect was totally abolished by a protease-activated receptor 1 inhibitor.

Conclusions—The prothrombotic phenotype of the SHR vessel wall was due to the ability of SMCs to support greater thrombin generation and resulted in accelerated occlusive thrombus formation after arterial injury, which was sensitive to glycoprotein Ibα–von Willebrand factor inhibitors. (Arterioscler Thromb Vasc Biol. 2015;35:930-937. DOI: 10.1161/ATVBAHA.115.305377.)

Key Words: arteries □ rats, inbred SHR □ smooth muscle cells □ thrombosis

The vascular wall is one of the elements that determines the thrombotic response.¹ Prior studies have shed light on the ability of vascular wall cells to support tissue factor (TF)–induced thrombin generation.²-⁴ We have recently demonstrated an increase in tissue factor pathway inhibitor (TFPI) and its secretion by cultured vascular smooth muscle cells (SMCs) in response to cyclic stress.⁵ It can be surmised that TFPI increases as vascular wall function deteriorates to compensate for a putative increase in its procoagulant potency.

It is still unclear whether hypertension causes a prothrombotic state in blood and within the vessel wall, although compelling clinical and experimental evidence points toward an impairment of the hemostatic balance in blood brought about by changes in individual coagulation factors in the presence of elevated blood pressure. In support of a hypercoagulable state, a few clinical studies have reported higher plasma levels of clotting factor VII, fibrinogen, and fibrin d-dimers in hypertensive patients than in normotensive subjects.⁶-⁸ In spontaneously hypertensive rats (SHR), in deoxycorticosterone acetate–induced hypertensive rats, and in endothelial dysfunction–induced experimental hypertension, a hypercoagulable state was demonstrated by elevated levels of thrombin–antithrombin complexes, as well as d-dimers, and supported by an increase in TF⁹ and a decrease in thrombomodulin.¹⁰ In experimental models of hypertension, the direct contribution of vascular cells to thrombin generation is not well documented. Early works focused primarily on the increased vascular expression of TF and antifibrinolytic molecules.⁹

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One concern is that the function of the complex thrombin generation process is difficult to assess from estimates of its separate contributing elements. It would be preferable to estimate the overall activity of the hemostatic and thrombotic system and its relation with vascular function. This can be attempted by using a global in vitro test, such as the calibrated automated thrombogram, designed to explore the thrombin generation process under conditions as close as possible to those in vivo. This test has been shown to indicate a thrombotic tendency in several clinical settings, which seems to be an independent predictor of acute ischemic stroke.

The present study tests, in SHR, the hypothesis that changes in coagulation proteins and membrane phospholipid organization cause an increased thrombin-generating capacity within the vascular wall because SMC phenotypic modulation is a hallmark of hypertension.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Hypertension-Associated Changes in Circulating Endothelial Markers

The weight of SHR rats was significantly lower compared with the age-matched Wistar rats (94±5 versus 141±2 g at 5 weeks of age and 335±5 versus 441±9 g at 12 weeks of age). We found that arterial blood pressure was already increased in SHR compared with Wistar at 5 weeks of age (156±9 versus 131±2 mm Hg, P=0.08). This difference was more pronounced at 12 weeks of age (174±1 versus 135±2 mm Hg, P<0.001). Perindopril treatment reduced arterial blood pressure and weight (Table I in the online-only Data Supplement). Although SHR exhibited a smaller systolic absolute diameter of the carotid artery, the ratio of systolic diameter to weight was increased in SHR compared with Wistar rats (Table I in the online-only Data Supplement).

To assess the degree of activation of the endothelium, we have measured plasma levels of von Willebrand factor (vWF) and TFPI in both Wistar and SHR rats. We found an increase in vWF (50±1% versus 34±2%, P=0.002) in 12-week-old SHR compared with age-matched Wistar. This difference was not observed in 5-week-old rats (45±3% versus 43±3%). TFPI was increased both in 5-week-old SHR (9.5±0.8 versus 6.4±0.5 U/mL, P=0.018) and 12-week-old SHR (6.9±0.2 versus 4.5±0.4 U/mL, P=0.005).

Aortic Rings From 12-Week-Old SHR Display Higher Thrombin-Generating Capacity Than Age-Matched Wistar Rings

When compared with 50 pmol/L TF alone (a concentration chosen to be close to levels of TF expressed by rings), addition of aortic rings from Wistar rats or SHR to a 12-week-old Wistar rat or SHR plasma pool significantly increased endogenous thrombin potential (ETP) values at 12 weeks (Figure 1B) but not at 5 weeks of age (Figure 1A). Analysis of the kinetics of thrombin formation and inactivation revealed a marked difference in peak height (Figure 1B). This procoagulant activity was significantly greater for SHR than for Wistar rings (Figure 1C). We also tested the effect of aortic rings of each animal in its own plasma and found the same increase in SHR.

To explore the contribution of SMCs, thrombin production was measured at the surface of either de-endothelialized or deadventitiated rings, as well as at the surface of rings after removal of both adventitia and endothelium. Thrombin generation was significantly increased in all rings from SHR compared with Wistar rings, and this procoagulant phenotype was reversed by perindopril treatment (Figure 1D).

When compared with Wistar rings, the higher procoagulant phenotype of 12-week-old SHR aortic rings seemed to
be unrelated to the expression of TF or TFPI activity because SHR rings expressed similar TF and TFPI activity as Wistar rings (Table). Comparison of TF activity associated with de-endothelialized or deadventitiated rings, as well as rings constituted of media alone, revealed that this activity originated mainly from the adventitia and the media (Table II in the online-only Data Supplement). However, the exposure of negatively charged phospholipids was ≈2-fold higher on SHR than on Wistar rings, and this increase was abrogated by perindopril treatment. As expected, such differences were not observed at 5 weeks of age.

**SMCs Are Responsible for the Procoagulant Phenotype of Aortic Rings**

When compared with endothelial cells (ECs) (CD31- and vWF-positive cells), SMCs (α-smooth muscle actin-positive cells), whatever their origin (12-week-old SHR or Wistar), displayed a greater ability to support thrombin generation. Figure 2A shows typical thrombin generation curves obtained with adherent SMCs and ECs in the presence of a Wistar platelet-free plasma pool. The times to thrombin burst and peak were significantly shortened and the peak, velocity index, and ETP values (Figure 2B) were higher for SMCs compared with ECs. Whereas no differences were observed in the thrombin generation patterns and parameters between SHR and Wistar ECs, a higher thrombin peak and ETP were obtained with SHR SMCs compared with Wistar SMCs. Thrombin generation at the surface of SMCs from 5-week-old SHR and Wistar rats was similar (ETP values were 525±18 and 559±20 nmol/L×min, respectively).

For both 12-week-old SHR and Wistar rats, TF activity was ≈20-fold higher in SMCs compared with ECs. SMC expression of TF and of TFPI was higher in SHR than in Wistar (Table). On the contrary, there was only a slight decrease in SMC expression of TF in 5-week-old SHR.

Measurement of phospholipid procoagulant activity revealed higher phosphatidylserine exposure on SHR than on Wistar SMCs at 12 weeks of age. We also examined the externalization of phosphatidylserine as a marker of apoptosis in SMCs using flow cytometry. Cells in early stages of apoptosis (annexin V-positive, propidium iodide–negative SMCs) were also increased in 12-week-old SHR compared with age-matched Wistar rats. These changes were not observed at 5 weeks of age.
Protease-activated receptor 1 (PAR-1) expression (Figure 3A) and Akt phosphorylation (Figure 3B) were significantly higher in SMCs from SHR than in those from Wistar rats at 5 and 12 weeks of age. To illustrate the cellular effects of thrombin, we investigated the proliferation of SMCs in response to thrombin (0.5 U/mL) in the presence or not of the PAR-1 antagonist SCH-79797 (100 nmol/L). Thrombin-induced proliferation was higher in SHR SMCs than in those from Wistar rats, and this difference was amplified at 12 weeks compared with 5 weeks of age (Figure 3C). Enhancement of SMC proliferation by thrombin was fully suppressed by the PAR-1 antagonist SCH-79797.

Hypertension-Mediated Vascular Changes Accelerate Thrombus Formation After Arterial Injury via Glycoprotein Ibα–vWF Interactions

In the absence of vascular injury, 12-week-old SHR did not have higher circulating thrombin–antithrombin complex levels than Wistar rats (49±10 versus 55±13 ng/mL) but exhibited a higher platelet count (527±14 versus 429±21×10^9/L, \( P=0.012 \)) and mean platelet volume (7.9±0.2 versus 6.6±0.1 flL, \( P=0.005 \)) without changes in platelet function (Figure II in the online-only Data Supplement). To characterize the effect of hypertension on arterial thrombosis in vivo, we triggered thrombosis by the application of ferric chloride to the carotid artery. The mean baseline blood flow was 520±48 mL/min (range, 279–857 mL/min) for Wistar rats and 436±37 mL/min (range, 190–645 mL/min) for SHR. SHR developed stable, occlusive thrombi, whereas embolization, indicated by successive decreases and increases in blood flow, was frequently observed in Wistar rats (Figure 4A). Occlusion was reached during the 30-minute observation period for 4 of the 11 Wistar rats and in 6 of 8 SHR (Figure 4B; \( P<0.05 \)), and the time to vessel occlusion was shorter in SHR than in Wistar rats (Figure 4C). No correlation was found between the carotid artery systolic diameter and the occlusion time. The hazard ratio for occurrence of occlusive thrombi in SHR was significantly higher: hazard ratio=4.418 (confidence interval 95%, 1.318–14.807), \( P=0.0161 \).

To test the involvement of glycoprotein Ibα (GPIbα)–vWF interactions in occlusive thrombosis and the thrombolytic efficiency of GPIbα–vWF inhibitors, aurintricarboxylic acid, which blocks the GPIbα binding domain of vWF, was injected 15 minutes after formation of a FeCl3-induced thrombus. Doppler flowmetry showed that aurintricarboxylic acid was effective in restoring vessel patency after occlusive thrombosis in SHR (Figure 4D). Conversely, aurintricarboxylic acid did not rescue blood flow in Wistar carotid arteries.

Discussion

This study demonstrates higher thrombin generation on the aortic wall because of increased procoagulant activities of SMCs from 12-week-old SHR. Our data show that established hypertension is required to induce increased thrombin generation within the vessel wall and subsequent SMC phenotypic changes. The main finding is that 12-week-old SHR exhibited accelerated FeCl3-induced thrombus formation in carotid arteries, and the resulting occlusive thrombi could be disaggregated by blockade of GPIbα–vWF interactions. Our study also highlights that hypertension-mediated SMC phenotypic modulation enhances PAR-1-dependent thrombin-induced SMC proliferation. Overall, our study extends the multifaceted pathophysiological actions of thrombin by demonstrating that enhanced thrombin formed on the surface of SMCs in hypertension increased arterial thrombosis after vascular injury and contributed to vascular remodeling.

Figure 2. Thrombin generation at the surfaces of spontaneously hypertensive rats (SHR) or Wistar smooth muscle cells (SMCs) and endothelial cells (ECs). Representative thrombin generation curves (A) and endogenous thrombin potential (ETP) values (B) in a Wistar platelet-free plasma (PFP) pool added to washed SMC or EC monolayers from 12-week-old rats. Results are means±SEM of 12 experiments; *\( P<0.05 \) vs Wistar.
Although thrombogenesis in hypertension has been shown to be mediated by endothelial dysfunction and platelet activation, increased thrombin generation has also been reported as a potential mechanism. From the hemodynamic point of view, the radial hydraulic conductance that occurs across the wall conveys soluble substances from the blood outwards. Because most clotting factors are of low molecular weight, it is likely that they will be easily conveyed from the lumen to the medial SMCs. Endothelial activation/dysfunction also plays a major role in exposing SMCs to clotting factors. Thrombin generation can be investigated either by measuring plasma markers of in vivo activation of coagulation or by assessing the reactivity of the clotting system, taking into account the role of vascular cells. Thrombin–antithrombin complexes levels were found previously to be elevated in SHR plasma. In the present study, thrombin–antithrombin complexes levels and thrombin generation in plasma were no higher in SHR than in Wistar, suggesting a vascular bed–specific effect in the hypertension-associated prothrombotic state. Our observation of an endothelial alteration indicated by elevated circulating levels of vWF in adult SHR compared with Wistar rats leads us to consider that vascular cells could enhance thrombin generation in SHR. To evaluate this specific contribution of the vessel wall to thrombin generation, aortic rings from SHR or Wistar were added to the same pool of plasma. We found that addition of 12-week-old SHR aortic rings to a Wistar or SHR plasma pool conferred a hypercoagulable state compared with Wistar rings. Although adding an aortic ring to plasma cannot fully mimic the physiological interactions between plasma and the vascular wall, this result can nevertheless be attributed to the vascular cells. Such a phenotype seems to be related to established hypertension because it was not observed at 5 weeks of age and was totally prevented by antihypertensive therapy. However, we cannot exclude a predominant role of angiotensin II because it has been shown that angiotensin II–induced hypertension is associated with accelerated thrombus development in SHR and not in deoxycorticosterone acetate-salt hypertension with low renin.

Our data demonstrated that ETP values in plasma were higher when adherent SHR rather than Wistar SMCs were used as cell surfaces, whereas no difference existed for ECs, suggesting a major contribution of SMCs in the coagulation phenotype of the arterial tissue. Our findings that thrombin generation was higher in the presence of de-endothelialized or deadventitiated rings or rings constituted of media alone from SHR support the conclusion that SMCs and not ECs account for the difference in thrombus development. Although it has been previously shown that SMCs have the capacity to secrete TF-enriched microparticles, under our experimental conditions thrombin generation at the surface of SMCs was unlikely to be influenced by such microparticles, because we used washed adherent cells. In addition, we found that SMCs support higher thrombin generation than ECs as reported previously.
The role of cells in coagulation is primarily to provide a negatively charged phospholipid surface for assembly of all of the actors of the coagulation cascade. Exposure of phosphatidylserine on the outer leaflet of platelets and vascular cells, regulated by a flip-flop mechanism, is thought to account for the procoagulant nature of cells. Our findings showing increased amounts of phospholipid procoagulant activity in aortic rings and SMCs from SHR provide a rationale to explain increased thrombin generation in SHR related to higher SMC negatively charged phospholipids. The reduced inhibiting effect of annexin V on thrombin generation at the surface of SMCs from 12-week-old SHR supports this hypothesis. The mechanisms for the increased levels of phospholipid procoagulant activity in SMCs from adult SHR may be related to the hypertension-induced elevation of intracellular calcium and the structural reorganization of cell membranes in SHR. The preventive effect of perindopril on SMC-supported thrombin generation is thus likely to be explained by a reduction of phospholipid procoagulant activity. It is unlikely that TFPI plays a major downregulating role in thrombin generation on SMCs in either strain because TFPI is known to dampen the burst of thrombin in response to a small procoagulant stimulus. The similar low inhibition of thrombin generation in presence of anti-TF antibodies does not argue for a major contribution of TF in the differences between SHR and Wistar rats. Because monocytes are inflammatory TF-bearing cells, macrophage infiltration in the subendothelium may contribute to the increased thrombin-generating capacity in SHR. However, such infiltration has been reported only in older SHR. Therefore, the involvement of macrophages in the subendothelium is unlikely at 12 weeks of age.

There is increasing evidence that specific binding proteins on membranes regulate the formation of coagulation complexes by localizing coagulation factors to cell surfaces. Increased integrin expression in the vessel wall of SHR has been reported previously. We can anticipate a synergistic effect between anionic phospholipid membranes, TF, and integrin on increased thrombin generation in the SHR vascular wall because this integrin is the receptor for prothrombin in SMCs and is involved in SMC-supported thrombin generation.

In this context, we would expect a difference in the incidence of thrombosis in Wistar and SHR and an exacerbation...
of the cellular effects of thrombin. The increase in thrombin generation within the vessel wall may modulate several well-known vascular processes involved in hypertension, such as the regulation of vascular tone and the proliferation of SMCs, leading to increased pulse pressure and arterial stiffening.18,20 The greater effect of thrombin on SMC proliferation in 5-week-old and 12-week-old SHR and its inhibition by SCH-79797 argue for enhanced cellular effects of thrombin contributing to vascular remodeling in hypertension. Our data indicate that the mechanism involves increased PAR-1 expression and Akt signaling pathway activation as previously reported.39

Thrombus formation in large arteries starts by platelet–platelet cross-linking involving GPIbIIa receptors and GPIbα–vWF interactions, followed by formation of a fibrin network to anchor the platelet-rich thrombus to the vessel wall.31 Fibrin formation is dependent on the exposure of procoagulant material on the SMC surface at the site of vascular injury. Most relevant to our study was the observation that hypertension-associated changes in procoagulant phospholipids at the outer surface of SMCs contributed to increased thrombin generation. The formation of occlusive thrombi and the marked shortening of the time to occlusion in SHR compared with Wistar rats suggest a critical role of increased SMC-supported thrombin generation in PAR-1–induced platelet activation and fibrin formation, both required for thrombus growth and stability. Although platelet count and PAR-1 expression were increased in SMCs from SHR, the absence of potentiated platelet function in SHR did not argue for a major contribution of PAR-1–mediated platelet activation to the differences in thrombus development between SHR and Wistar rats. Recent data have highlighted the role of GPIbα–vWF interactions during vessel lumen closure and revealed that disruption of these interactions restores vessel patency after occlusive thrombosis but with a limited therapeutic window.32,33 Whereas fully occlusive thrombi in SHR were efficiently disaggregated by GPIbα–vWF inhibitors, nonocclusive thrombi in Wistar rats were resistant. This absence of restoration of Wistar rat vessel patency is consistent with a hierarchical process of occlusive thrombosis in which platelet–platelet cross-linking in the inner core mainly involves GPIbIIa, whereas once the thrombus has occluded 50% of the artery, platelet cross-linking in the external layer occurs in a GPIbα–vWF–dependent manner.32,33 Although it is now increasingly recognized that multiple concomitant mechanisms regulate the formation and stability of arterial thrombi, our study further points to the attractiveness of this GPIbα–vWF axis as a potential therapeutic target for occlusive thrombosis in the context of hypertension. Whether coadministration of an inhibitor of GPIbα–vWF interactions and a specific inhibitor of integrin αvβ3 to target thrombin generation on the surface of SMCs may lead to the development of more efficient therapeutic strategies remains to be investigated.

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Disclosures
None.

References
receptor 1. Thrombin generation within the vessel wall may also exert proliferative effects on smooth muscle cells which involve the protease-activated receptor 1. This results in accelerated occlusive thrombus formation after arterial injury. Restoration of vessel patency by blockade of glycoprotein Ibα-VWF blockade restores vessel patency by dissolving platelet aggregates formed under very high shear rate in mice. Blood. 2014;123:3354–3363. doi: 10.1182/blood-2013-12-543074.


**Significance**

Smooth muscle cell plasticity is a hallmark of hypertension, and pulsatile hemodynamics play a crucial role in the production and secretion of procoagulant and anticoagulant factors by vascular smooth muscle cells. Our findings demonstrate that established hypertension induced increased thrombin generation on the surface of vascular smooth muscle cells by promoting negatively charged phospholipid exposure and increasing synthesis of tissue factor. This results in a prothrombotic phenotype of the arterial tissue in adult spontaneously hypertensive rats resulting in accelerated occlusive thrombus formation after arterial injury. Restoration of vessel patency by blockade of glycoprotein Ibα-VWF or von Willebrand factor interactions supports the potential beneficial effect of such inhibitors in occlusive thrombosis. Hypertension-mediated thrombin generation within the vessel wall may also exert proliferative effects on smooth muscle cells which involve the protease-activated receptor 1.
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Material and methods

Animals
Five week-old and 12 week-old, male spontaneously hypertensive rats (SHR) and Wistar rats were obtained from Charles River (France). All of the experiments complied with the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health and all procedures were approved by the Animal Ethics Committee of the Institut National de la Santé et de la Recherche Médicale. Systolic blood pressure was measured using a tail-cuff blood pressure analyzer (Blood Pressure Analysis System, model SC-1000; Hatteras Instruments, Cary, NC) on conscious animals. Systolic diameter and thickness of the right common carotid artery were measured by echography using a Vevo 770 High-Resolution Imaging Systems (VisualSonics Inc., Amsterdam, The Netherlands). A group of 8 SHR rats were treated with the angiotensin II-converting enzyme inhibitor, perindopril, in the drinking water (60 mg/L) from 5 weeks of age for 7 weeks.

Aortic rings and cells
The descending thoracic aorta was excised from rats after isoflurane anesthesia (4.5% in 1.5 L/min dioxygen) and exsanguination. Two-millimeter ring segments were prepared from a first set of 18 Wistar rats, 18 SHR and 8 treated SHR and washed for 1 h at 4°C with 20 mM HEPES, 140 mM NaCl, pH 7.35 containing 5 g/L albumin (HBS) and then used for thrombin generation assays or phospholipid procoagulant activity. Aortic smooth muscle cells (SMCs) and endothelial cells (ECs) were isolated from a second set of 8 SHR and 8 Wistar rats at each age as previously described. SMCs were grown in DMEM/F12 supplemented with 10% foetal bovine serum and ECs in Clonetics® EGM®-2 Endothelial Cell Growth Medium-2 (Lonza, Basel, Switzerland) supplemented with 10% horse serum. For thrombin generation assays, SMCs at passages 3-5 or ECs at passage 2 were seeded (7500 cells/well) in 96-well tissue culture flat-bottom plates (MICROTEST™96), grown to subconfluence and washed with HBS before use.

Blood collection and plasma preparation
Rats were anesthetized with isoflurane and whole blood was collected via a carotid catheter into syringes containing one-tenth the volume of 0.106 M sodium citrate. Blood was centrifuged at 194g for 10 min at room temperature to obtain platelet-rich plasma (PRP) and then at 1750g for 10 min to obtain platelet-poor plasma. PRP was adjusted to 500×10^9 platelets/L by addition of autologous platelet-poor plasma. Platelet-free plasma (PFP) was obtained by centrifugation of platelet-poor plasma at 13000g for 30 min at 4°C, and frozen at −80°C.

Hematologic analyses
Complete blood counts and haematocrit were determined with an automatic cell counter (Micros 60 ABX model, Montpellier, France). Circulating von Willebrand factor (vWF) antigen was measured by ELISA (Asserachrom®, Diagnostica Stago, Asnières, France). Each plasma sample was assayed at two different dilutions chosen in order to interpolate results, expressed as percentages, using the calibration curve obtained with the human calibrator supplied within the kit. Thrombin-antithrombin complexes were measured with the Enzygnost® TAT micro ELISA kit (Instrumentation Laboratory, Paris, France). Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) activities were measured in 200 µL HBS incubated for 1 h at room temperature with aortic rings using the Actichrome® tissue factor and Actichrome® TFPI activity assay respectively (American Diagnostica, Stamford, CT).
measure cell surface TF or TFPI activity, washed adherent SMCs or ECs were overlaid with 75 and 20 µL of HBS respectively. The reaction was stopped with 50 µL of glacial acetic acid and the absorbance was read at 405 nm. Protease-activated receptor 1 expression was measured by an ELISA kit (USCN Life Sciences, Wuhan, China) in SMC proteins obtained by lysing SMCs in complete Lysis-M buffer (Roche Diagnostics Corporation, Basel, Switzerland). An amount of 20 µg of total proteins was added to each well.

**Thrombin generation assays**

Calibrated automated thrombography was performed at 37°C in a microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using a dedicated software program (Thrombinoscope BV, Maastricht, The Netherlands) as reported previously. Thrombin generation was triggered by recalcification in the presence of recombinant human TF (Dade Behring, Marburg, Germany). Round-bottom 96-well Greiner blue plates were used for PFP in which aortic rings had been added, and MICROTEST™96 plates for SMC or EC monolayers. Thrombin generation curves were recorded in triplicate and the endogenous thrombin potential (ETP) was assessed as the area under the curve.

**Phospholipid Procoagulant Activity**

The chromogenic assay measuring the phospholipid-related procoagulant activity (PPA) in aortic rings was performed as described previously for plasma. Rings resuspended in 50 µL of 50 mM Tris, 175 mM NaCl, pH 7.9 (TBS) containing 2 g/L bovine serum albumin (BSA) were added to wells containing 50 µL of activated factor X (FXa) (1.2 nM), activated factor V (FVa) (2.4 nM), CaCl₂ (15 mM) and 50 µL of bovine prothrombin (6 µM) plus S2238 substrate (0.6 mM). The absorbance change was read at 405 nm at 37°C. For SMC monolayers, washed adherent cells were incubated with 50 µL of TBS-BSA, 50 µL of FXa-FVa-CaCl₂ and 50 µL of bovine prothrombin (6 µM) plus Z-Gly-Gly-Arg-AMC substrate (1.25 mM) in 20 mM HEPES pH 7.5 containing 60 g/L BSA. The plate was placed in the Fluoroskan Ascent fluorometer and allowed to warm to 37°C for 5 min before kinetic readings were taken over 10 min. Phospholipid concentration was estimated from the initial rate of thrombin formation by reference to a standard curve constructed with a mixture of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (20:20:60 mole%), and expressed as PS equivalents.

**Washed platelet preparation**

Whole blood was collected via a carotid catheter into syringes containing one-sixth the volume of an acid-citrate dextrose solution. Blood was centrifuged at 190g for 4 min followed by 70 seconds at 1900g at room temperature to obtain PRP and then platelets were sedimented by centrifugation at 5000g for 4 min. Platelets were resuspended at a final concentration of 2×10⁸/mL in Tyrode buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, pH 7.3).

**Platelet aggregation**

Platelet aggregation was measured by turbidimetry at 37°C under stirring conditions in presence of 1.27 mg/mL exogenous rat fibrinogen. Washed platelets (2×10⁹/mL) were stimulated by 5 µg/mL collagen (SD Innovation, Frouard, France) or 10 µM ADP (SD Innovation) or 10 nM thrombin (Diamed), and aggregation was monitored for 10 min using a TA-8V aggregometer (SD Innovation).

**Flow cytometry**

Washed platelets (2×10⁹/mL) were incubated with increasing concentrations of collagen or thrombin for 15 min at 37°C. Non-stimulated and stimulated platelets (5 µL) were incubated with a Phycoerythrin (PE)-conjugated anti-P-selectin antibody (Santa Cruz) in a final volume of 50 µL PBS. Phosphatidylserine exposure was quantified by annexin V labelling using
FITC-coupled annexin V (Beckman Coulter, Villepinte, France). Samples were analysed using a Gallios flow-cytometer (Beckman Coulter). Apoptosis was examined by a double staining method using the fluorescein-isothiocyanate (FITC)-labelled annexin V/propidium iodide (PI) apoptosis detection kit (Beckman Coulter) according to the manufacturer’s instructions. Briefly, trypsinized SMCs were washed twice with PBS, and stained with FITC-conjugated annexin V and PI dyes. The externalization of PS and the permeability to PI were evaluated by flow cytometry on a Gallios flow-cytometer. Data from 10,000 events per sample were collected. Cells in early stages of apoptosis were positively stained only with annexin V.

**Western blotting**
Proteins were obtained by lysing SMCs in complete Lysis-M buffer (Roche Diagnostics Corporation, Basel, Switzerland). Lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes and incubated with antibodies against α-tubulin (Sigma), Akt, and phosphorylated-Akt (Cell Signalling, Danvers, MA). Immune complexes were detected with appropriate secondary antibodies and enhanced chemiluminescence reagent (Bio-Rad, Marnes la Coquette, France).

**Cell proliferation assay**
SMCs were seeded at a density of $10^4$ cells/well in 24-well plates. At 60-80% confluence, cells were washed with PBS and then starved for 24 h in DMEM/F12 without serum. Medium was then replaced with fresh serum-free DMEM/F12 containing 0.5 U/mL bovine thrombin (Synapse B.V., Maastricht, The Netherlands), with or without the protease-activated receptor 1-selective antagonist SCH-79797 (100 nM) for 24 h. The 0.5 U/mL concentration represented half of the level required for clot formation (1 U/mL) and was thus selected to mainly elicit the mitogenic effect of thrombin. Cells were counted after trypsinization in a Scepter™ 2.0 Handheld Automated Cell Counter (Merck Millipore, Molsheim, France).

**Ferric chloride-induced carotid artery thrombosis model**
Rats were anesthetized with 2% isoflurane in 0.5 L/min oxygen and the right common carotid artery was exposed by blunt dissection. A $5 \times 10$ mm filter paper soaked in freshly prepared 10% ferric chloride (Sigma-Aldrich, St Louis, USA) was applied to the surface of the adventitia of the exposed artery for 3 minutes. After removal of the filter paper, the artery was washed with 0.9% NaCl and blood flow was monitored for 30 min via Doppler transonic flow probe (RMV707B, 15-45 MHz, FUJIFILM Visualsonics Inc, Amsterdam, The Netherlands). Occlusion time was defined as the time between FeCl₃ application and blood flow decrease to less than 20 mL/min for more than 1 min.
To study the role of glycoprotein (GP)Ibα-vWF interactions, some rats were injected intravenously with aurintricarboxylic acid (ATA, 20 mg/kg; Sigma-Aldrich), a polycarboxylated compound which binds to the A1 domain of vWF, thus preventing binding of vWF to platelet Gplbα. ATA was injected as a rapid bolus 15 min after occlusive thrombosis.

**Statistical analysis**
Results are expressed as means ± SEM. Comparisons between groups at the same age were performed using the Wilcoxon rank test. The percentages of rats with patent arteries in each group are plotted in a Kaplan–Meier graph as a function of time after initiation of FeCl₃ injury. A Cox proportional hazard model was used to compare groups. Differences were considered statistically significant at $P < 0.05$. 
References for methods


Supplemental Table I: Vascular parameters

<table>
<thead>
<tr>
<th></th>
<th>Wistar</th>
<th>SHR</th>
<th>Treated SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>441 ± 9</td>
<td>335 ± 5 *</td>
<td>284 ± 5 *#</td>
</tr>
<tr>
<td>Conscious SBP (mmHg)</td>
<td>135 ± 2</td>
<td>174 ± 1 *</td>
<td>117 ± 3 *#</td>
</tr>
<tr>
<td>Systolic CA thickness (µm)</td>
<td>179 ± 9</td>
<td>181 ± 7</td>
<td>161 ± 10</td>
</tr>
<tr>
<td>Systolic CA diameter (µm)</td>
<td>1141 ± 21</td>
<td>968 ± 28 *</td>
<td>835 ± 75 **#</td>
</tr>
<tr>
<td>Systolic CA diameter/weight (µm/g)</td>
<td>2.60 ± 0.06</td>
<td>2.91 ± 0.11 *</td>
<td>2.96 ± 0.29</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure, CA, carotid artery.

Values are means ± SEM. * P<0.05 vs Wistar. ** P<0.05 vs SHR.
**Supplemental Table II:** Tissue factor activity associated with de-endothelialized or de-adventitiated rings or rings constituted of media alone

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Wistar (n=6)</th>
<th>SHR (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-endothelialized rings</td>
<td>97 ± 10</td>
<td>94 ± 14</td>
</tr>
<tr>
<td>De-adventitiated rings</td>
<td>19 ± 3</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Rings constituted of media alone</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
Figure I. **Effect of a polyclonal anti-tissue factor antibody or annexin V on thrombin generation at the surface of SMCs.** The antibody (10 µg/mL), or annexin V (5 µmol/L), was added prior to the addition of 16.7 mM CaCl₂. Results are expressed as the ratio between values in the presence and those in the absence of anti-tissue factor antibody (left panel) or annexin V (right panel). Data represent means ± SEM of SMC cultures from 3 different rats, tested in triplicate. * P<0.05 vs Wistar at the same age.
Figure II. Platelet function in SHR and Wistar rat. (A) Representative aggregation tracings and mean maximum aggregation in response to various agonists (5 µg/mL collagen, 10 µM ADP and 10 nM thrombin) in the presence of 1.27 mg/mL fibrinogen. (B) Representative flow cytometry histograms and mean annexin V binding to non-stimulated washed platelets or washed platelets incubated with various concentrations of thrombin or collagen. (C) Representative flow cytometry histograms and mean percentages of P-selectin-positive cells of non-stimulated washed platelets or washed platelets incubated with various concentrations of thrombin or collagen. Results are presented as means ± SEM (n=11 for Wistar rats and SHR and n=8 for treated SHR).