Original Article

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Impact of angiotensin-converting enzyme inhibition on platelet tissue factor expression in stroke-prone rats

Marta Brambilla[a,*], Paolo Gelosa[a,*], Laura Rossetti[a,*], Laura Castiglioni[b], Chiara Zara[a], Paola Canzano[a], Elena Tremoli[a,b], Luigi Sironi[a,b], and Marina Camera[a,b]

Objective: Hypertension is a well known risk factor for thrombotic events such as myocardial infarction and stroke. Platelets express tissue factor (TF), the key activator of blood coagulation and thrombus formation. The number of TF-positive platelets increases in pathological conditions characterized by thrombotic complications but whether this occurs in hypertension is unknown. Here we investigated whether platelet TF expression is increased in a hypertensive status through a mechanism acting on megakaryocytes; the phenomenon could be modulated by antihypertensive drug as captopril; angiotensin (AngII) influences platelet TF expression.

Methods: Spontaneously hypertensive stroke prone (SHRSP) rats received standard diet (StD) or a Japanese high-salt permissive diet (JpD). After 3 weeks, JpD animals were randomized to receive captopril or vehicle. Normotensive Wistar Kyoto (WKY) rats were used as controls. Cell-associated TF expression and activity were analyzed by flow cytometry and calibrated automated thrombogram, respectively.

Results: Hypertensive StD-SHRSP showed an increased number of TF-positive platelets compared with normotensive WKY. After JpD administration, SHRSP developed severe hypertension and renal damage; the number of TF-positive megakaryocytes significantly increased compared with StD-SHRSP resulting in a higher number of TF-positive platelets with a faster kinetic of thrombin generation. These effects were reverted by captopril. Ex-vivo stimulation of platelets, isolated from normotensive WKY and from healthy individuals, with AngII induced a concentration-dependent increase of surface-associated TF expression.

Conclusion: The current study shows for the first time that in hypertension the number of TF-positive megakaryocytes increases thus releasing in the circulation more platelets carrying a functionally active TF. AngII stimulates platelets to express TF.

Keywords: angiotensin II, antihypertensive drug, blood pressure, platelets, stroke-prone rats, tissue factor

Abbreviations: ACE, angiotensin-converting enzyme; AngII, angiotensin; JpD, Japanese high-salt permissive diet; SHRSP, spontaneously hypertensive stroke prone rats; StD, standard diet; TF, tissue factor; WKY, Wistar Kyoto rats

INTRODUCTION

Hypertension is a well known risk factor for cardiovascular diseases [1]. Indeed it has been linked with a prothrombotic state characterized by platelet activation and increased levels of circulating as well as monocyte-associated tissue factor (TF) expression [2].

TF is the main activator of the blood coagulation cascade, and it has been shown by us and others to be expressed also by human platelets, which transfer it on their cell surface upon activation [3–5]. More recently, we have provided evidence that TF behaves as an endogenously synthesized protein that characterizes megakaryocyte maturation and that it is transferred to a subset of newly released platelets. Platelet TF is functionally active and able to trigger thrombin generation, thus contributing to the global platelet hemostatic capacity [6].

The number of TF-positive platelets increases in pathological conditions such as in patients with cancer, with polycythemia vera and essential thrombocythemia, and with acute coronary syndrome [7–9]. Among the cardiovascular risk factors, diabetes has been shown to affect platelet TF expression both in human and in rat platelets [10,11]. Of interest, in a recent study carried out to elucidate the hypoxia-induced prothrombotic phenotype, the proteomic analysis of platelets isolated from rats exposed to hypoxic stimulus showed that TF was one of the differentially expressed proteins (increased expression compared with controls), thus potentially contributing to the thrombotic risk associated with a hypoxic environment [12].

Whether platelet TF expression is affected in hypertension is so far a missing information. Thus, to address this

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[a]Centro Cardiologico Monzino IRCCS and [b]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

Correspondence to Prof. Marina Camera, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti, 9, 20133 Milan, Italy. Tel: +39 02 58 00 22 55; fax: +39 02 58 00 26 23;
e-mail: marina.camera@unimi.it, marina.camera@ccfm.it

*Marta Brambilla, Paolo Gelosa and Laura Rossetti contributed equally to the article.

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issue, we took advantage from the spontaneously hypertensive stroke prone (SHRSP) rats, a strain which, among the available hypertensive rat models, most recapitulates the evolution of human essential hypertension, gradually developing renal injury and ultimately also stroke [13,14]. In these animals, we investigated, by whole blood flow cytometry, whether a condition of hypertension and also organ damage affect platelet TF expression and whether a mechanism acting on megakaryocytes at the bone marrow level could be involved.

In the setting of a severe hypertensive status, characterized by a marked organ damage, we also evaluated the effect of captopril on platelet and megakaryocyte TF expression.

Finally, as angiotensin (AngII), which is significantly increased in severe hypertensive SHRSP rats [15,16], induces TF expression in monocytes, in endothelial cells and in smooth muscle cells [17–19] we evaluated, by means of ex-vivo experiments, its effect on platelet TF expression.

METHODS

Experimental plan

The procedures concerning animal care and euthanasia were carried out in accordance with national and international laws and policies, and authorized by the Ministry of Health – Committee of Università degli Studi di Milano (approval number 12/12-30012012).

The flowchart of this study is described in Fig. 1. In details, 7-week-old male SHRSP ($n = 33$, Charles River, Wilmington, Massachusetts, USA) were randomly divided to be fed with a standard diet (StD; $n = 10$) or with a Japanese high-salt permissive diet (JpD; $n = 23$) containing 18.7% proteins, 0.63% potassium and 0.37% sodium (Laboratorio Dottori Piccioni, Gessate, Milan, Italy) and received 1% NaCl in drinking water [14]. After 3 weeks, when a marked increase of blood pressure (BP) levels together with the onset of renal damage was induced by JpD, consistent with our previous study [14], JpD-SHRSP rats were randomized to receive or not captopril (50 mg/kg per day; Sigma-Aldrich, St Louis, Missouri, USA) dissolved in drinking water (JpD-cap; $n = 10$ and JpD; $n = 13$). To be sure of completeness of the administration, captopril was dissolved in 5 ml of drinking water and administered to each rat. After the ingestion of the full dose of the drug, drinking water and 1% NaCl was available to each animal *ad libitum*.

Seven-week-old male Wistar Kyoto (WKY; $n = 15$) rats were chosen as normotensive control animals and randomly assigned to be fed with standard diet (StD-WKY; $n = 10$) or with Japanese permissive diet (JpD-WKY; $n = 5$).

Systolic arterial BP measurements were performed at baseline (week 0) and after 3, 4 and 8 weeks, in conscious rats using a tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, North Carolina, USA). Rats were conditioned to the restraint and the warming chamber for 10–20 min/day for at least 2 days prior to measurements. After this training period, BP was measured. Following the same time schedule, the animals were housed in individual cages for 24 h for urine collection and proteinuria evaluation.

At week 3, a group of JpD-SHRSP ($n = 3$) was sacrificed and kidney was processed for histological analysis as control of the illness severity stage before captopril administration. As the aim of the study was to characterize the platelet activation in hypertensive state, avoiding that due to a stroke event [20], all the other ($n = 10$) severe hypertensive SHRSP animals (JpD) were sacrificed after 4 weeks from the beginning of the study. Within this timing, based

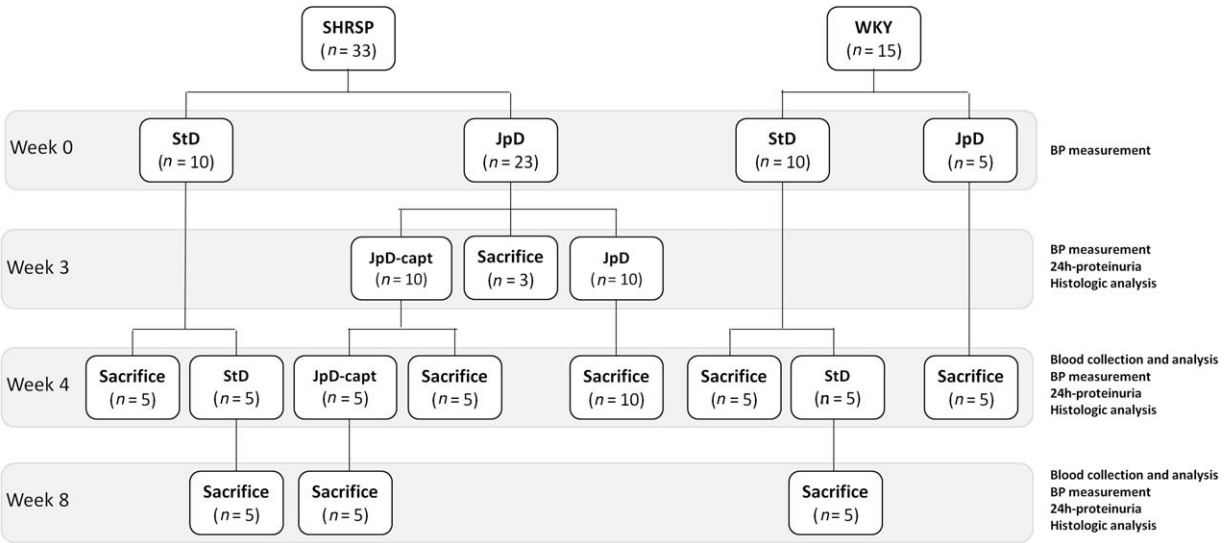

FIGURE 1 Outline of the experimental protocol. Spontaneously hypertensive stroke prone ($n = 33$) and Wistar Kyoto ($n = 15$) rats were randomly divided to be fed with a standard diet or with a Japanese high-salt permissive diet. Three weeks after the beginning of the study, Japanese high-salt permissive diet-spontaneously hypertensive stroke prone rats were randomized to receive or not captopril (50 mg/kg per day) dissolved in drinking water (Japanese high-salt permissive diet-cap, $n = 10$; Japanese high-salt permissive diet, $n = 13$). At the time of randomization, a group of Japanese high-salt permissive diet-spontaneously hypertensive stroke prone ($n = 3$) was sacrificed and kidney was processed for histological analysis. To avoid the onset of stroke events, 4 weeks from the beginning of the study all the other Japanese high-salt permissive diet-spontaneously hypertensive stroke prone were sacrificed together with Japanese high-salt permissive diet-Wistar Kyoto and half of the number of standard diet-Wistar Kyoto, Japanese high-salt permissive diet-cap and standard diet-spontaneously hypertensive stroke prone rats for data comparison. The remaining animals were sacrificed after 8 weeks.

on our previous experience [14], no stroke event occurred, as confirmed by brain T2-weighted MRI analysis [21] performed on all animals before sacrifice. JpD-WKY and half of the number of StD-WKY, of JpD-cap and StD-SHRSP rats were sacrificed together with the JpD-SHRSP animals for data comparison, whereas the remaining animals were sacrificed after 8 weeks.

Blood and bone marrow collection

Four and 8 weeks from the beginning of the study, blood was drawn from the tail vein with a 23-ga needle into citrate (1/10 volume of 0.129 mol/l sodium citrate) and corn trypsin inhibitor (50 μg/ml) – containing tubes. Blood cell counts were determined with the Sysmex XT-2000iV Hematology Analyzer (Dasit, Cornaredo, Milan, Italy). For platelet isolation, whole blood was diluted (five-fold) in saline solution (0.9-g/l NaCl) and centrifuged at $75 \times g$ for 15 min at room temperature (RT) with brake off. Platelet-rich plasma (PRP) was then centrifuged at $10\,000 \times g$ for 5 min and platelet pellets stored at −80 °C until used. Mononuclear leukocytes were separated by centrifugation of platelet depleted blood on a Ficoll-Paque density gradient (GE Healthcare Life Sciences, Chicago, Illinois, USA), according to manufacturer's instructions, and stored at −80 °C until used.

At sacrifice, femurs from four rats for each experimental group were isolated to collect megakaryocytes. Briefly, bone marrow was flushed into PBS containing 1/10 volume of 0.129-mol/l sodium citrate and 1-mmol/l EDTA and single-cell suspensions used for flow cytometry analysis.

Histological analysis

Paraffinized slices (5 μm) of renal tissue fixed in 10% formalin (Merck, Germany) were used for staining with hematoxylin/eosin. Tubular atrophy and luminal cast formations were assigned to a separate scores ranging from 0 to 3 (absent, mild, moderate and severe changes), and the scores were combined to obtain a mean tubular damage score, as previously described [22].

At least 1000 tubules were examined from each animal ($n = 3$ rats for JpD-SHRSP at 3 weeks; $n = 5$ rats for all other groups).

Flow cytometry

Tissue factor expression was analyzed by flow cytometry on 1% paraformaldehyde fixed whole blood or bone marrow. For intracellular staining, cells were permeabilized for 10 min with a 0.1% Triton X 100-PBS solution. Surface and intracellular antigen staining were performed as follows: 2.5 μl of samples were incubated for 15 min, in the dark, at RT, with saturating concentration of centrifuged ($17\,000 \times g$, 5 min, 4 °C) rabbit anti-TF antibody (1.5 μg/sample, Abcam, Cambridge, UK). Samples were centrifuged at $1500 \times g$ for 5 min and incubated with goat anti-rabbit Alexa Fluor 633 labeled IgG (1 : 200, Thermo Fisher, Waltham, Massachusetts, USA). After a washing step, phycoerythrin-conjugated anti-CD61 (500 ng/sample, Abcam) and fluorescein-isothiocyanate conjugated anti-CD45 (100 ng/sample, Abcam) antibodies were added.

Fluorochrome-conjugated isotype or Alexa Fluor 633-labeled IgG controls were used in all the experiments to quantify the background labeling. A total of 10 000 CD61-positive or 5000 CD45-positive events per sample were acquired on a BD FACSCalibur. Cytometer performances were checked by daily running BD CompBeads. Data are reported as percentage of cells positive for the analyzed antigens or as mean fluorescence intensity. All the data were analyzed with DIVA Software (BD Biosciences, San José, California, USA).

Imaging flow cytometry

Imaging flow cytometry was performed on ImageStream^X MarkII (Amnis, Seattle, Washington, USA). Bone marrow fixed cells were stained with phycoerythrin-conjugated anti-CD61 (500 ng/sample, Abcam), Alexa488 labeled anti-TF antibody (1.5 μg/sample, Abcam) and with DRAQ5 (5 μmol/l, Abcam), a fluorescent DNA dye used for nuclei identification.

Events were acquired with the 20× objective, at low flow rate/high sensitivity. Data analysis was performed with IDEAS software (version 6.1; Amnis). Megakaryocytes were analyzed by gating on CD61-positive and focused events, with an area higher than 1000 μm^2. Cell aggregates were excluded from analysis by selecting only the single-cell population defined as events with an aspect ratio (minor axis/major axis of a selected event) higher than 0.6. Images were compensated based on a matrix generated by single-stained samples acquired with identical laser settings in the absence of brightfield illumination.

Thrombin generation assay

The thrombin generation capacity of platelets was analyzed on cell lysates. Cells, isolated as described above, were solubilized with 15-mmol/l octyl-β-D-glycopyranoside at 37 °C for 15 min, sonicated for 1 min, diluted with 25-mmol/l HEPES–saline buffer and tested for their capacity to promote thrombin generation using the calibrated automated thrombogram (CAT) assay. An amount of 40 μl of platelet lysate (containing 4 μg of proteins) was incubated for 10 min with 40 μl of platelet-free normal pooled plasma (Pool Norm; Stago, Maastricht, The Netherlands) in round-bottom 96-well microtiter plates (Immulon 2HB). Thrombin generation was triggered by the addition of 20 μl of $CaCl_2$/fluorogenic substrate mixture (FluCa Kit; Stago) and fluorescence was read for 60 min in a Fluoroskan Ascent reader (Thermo Labsystems, Philadelphia, Pennsylvania, USA) equipped with a 390/460 filter set. Samples devoid of cell lysates were run to set the background level of thrombin generated by the assay. To this aim, 40 μl of platelet-free normal pooled plasma (Pool Norm; Stago) were incubated with 40 μl of octyl-β-D-glycopyranoside/HEPES–saline buffer. To assess the TF-dependent contribution to thrombin generation, the CAT assay was performed after preincubation of the samples with a neutralizing anti-TF antibody (100 μg/ml, cat 4501; Sekisui Diagnostics, Lexington, Massachusetts, USA). To correct for inner filter effects and substrate consumption, all thrombin generation measurements were calibrated against the fluorescence curve obtained after the addition of fixed amount of thrombin-α2-macroglobulin complex (Thrombin Calibrator; Stago). Thrombin generation curves were analyzed by dedicated software (Thrombinoscope BV). Lag time

(min), peak height (nmol/l thrombin) and the velocity index (nmol/l thrombin/min) were used as main parameters describing thrombin generation.

Ex-vivo stimulation of rat and human platelets with angiotensin

For ex-vivo studies, blood from normotensive WKY rats ($n = 6$) was drawn from the tail vein and PRP was prepared as described above. A total of 1×10^6 platelets were stimulated with collagen (1 and 4 μg/ml) or selective AT1 agonist (Val5AngII) (1, 10 and 100 nmol/l, Santa Cruz, Dallas, Texas, USA) [23], for 5 min, at 37 °C, under stirring condition. Preincubation with valsartan (500 nmol/l, Sigma-Aldrich), an AT1 antagonist, was carried out for 2.5 min before cell stimulation. Platelet TF expression was analyzed by flow cytometry as reported above.

For experiments on human platelets, venous blood was collected from healthy individuals ($n = 3$) who did not take antiplatelet drugs within 10 days before blood donation and who gave their informed consent to participate in the study. After the first 4 ml were discarded, whole blood was drawn with a 19-ga needle without venous stasis into citrate-containing tubes (1/10 volume of 0.129 mol/l sodium citrate vacutainer; Becton Dickinson, Franklin Lakes, New Jersey, USA). PRP was prepared by whole blood centrifugation at $100 \times g$ for 10 min, with brake off, and platelet count was determined with the Sysmex XS-1000i Hematology Analyzer (Dasit). Platelets (1×10^6) were stimulated with increasing concentration of AngII (100 pmol/l–100 nmol/l, Sigma-Aldrich), for 5 min, at 37 °C, under stirring condition, in the presence of saturating concentrations of MoAb against human TF (cat 4507CJ, Sekisui) and human GpIIb (CD41; Beckman Coulter, Brea, California, USA). Platelet surface TF expression was analyzed by flow cytometry as previously described [9].

Statistical analysis

Data were expressed as the mean ± SD. General linear models (GLM) were applied to evaluate, on all dependent variables, the effect of rat strain and diet and their interaction. Post-hoc tests, with Bonferroni correction, were performed to assess differences between specific combinations of strain and diet. Another GLM was applied to evaluate the effect of captopril in SHRSP rats. The effect of captopril treatment was analyzed as a trend according to treatment duration and post-hoc tests with Bonferroni correction were performed between specific treatments (no captopril, 1-week treatment and 5-week treatment).

In ex-vivo experiments, analysis of variance with post-hoc tests was used to compare independent groups. Analyses were performed using SAS V9.4 statistical package (SAS Institute, Cary, North Carolina, USA). A P value less than 0.05 was considered statistically significant.

RESULTS

Effect of Japanese diet and captopril treatment on SBP and renal organ damage

The mean values of the systolic arterial BP as well as the 24-h proteinuria levels of the animals included in the study are summarized in Tables 1 and 2, respectively.

TABLE 1. Rat systolic arterial blood pressure values

Pressure (mmHg)	Week 3	Week 4	Week 8
StD-SHRSP	180 ± 14*	183 ± 21	184 ± 9
JpD-SHRSP	229 ± 19**	233 ± 17***	ND
JpD-cap	ND	216 ± 20	202 ± 16****
StD-WKY	144 ± 9	ND	ND
JpD-WKY	153 ± 9	ND	ND

Data are mean (mmHg) ± SD. General linear models were applied for statistical analysis. JpD, Japanese high-salt permissive diet; ND, not determined; SHRSP, spontaneously hypertensive stroke prone; StD, standard diet; WKY, Wistar Kyoto.
*$P = 0.0001$ StD-SHRSP vs. StD-WKY.
**$P < 0.0001$ StD-SHRSP vs. JpD-SHRSP.
***$P = 0.001$ StD-SHRSP vs. JpD-SHRSP.
****$P < 0.05$ JpD-cap vs. JpD-SHRSP by post-hoc test adjusted by Bonferroni correction.

For all the variables, the effect of rat strain and diet as well as their interactions are reported in the Online Supplementary Table S1, http://links.lww.com/HJH/A906.

At the beginning of the study, BP of SHRSP rats fed with the StD was already significantly higher compared with StD-WKY (166 ± 14 vs. 143 ± 7 mmHg, respectively; $P = 0.003$). It slightly increased after 3 weeks remaining unchanged until the end of the study. The urinary protein excretion in this animal group was constant throughout the study period and comparable to that of StD-WKY, indicating that no renal damage occurred in this hypertensive strain fed with a StD, as also confirmed by the histological analysis of the kidney (Fig. S1, http://links.lww.com/HJH/A906).

Conversely, administration of high-salt diet (JpD) for 3 weeks induced severe hypertension in SHRSP rats with a concomitant significant increase in urinary protein excretion. At week 4, no further change in BP was recorded, whereas a ~17-fold higher proteinuria level compared with StD-SHRSP rats was measured. The establishment of the severe renal damage in JpD-SHRSP rats was confirmed by the histology of the kidney (Fig. S1, http://links.lww.com/HJH/A906). At week 3, JpD-SHRSP rats showed a mild vascular alterations characterized by the presence of few atrophic tubules. One week later, the renal tissue structure worsened and severe tubular damage and luminal cast formation significantly increased.

Of note, JpD did not induce significant variation of BP and of proteinuria levels in WKY animals. Indeed, the kidney of JpD-WKY animals showed no relevant histological abnormalities.

Captopril administered for 1 week to JpD-SHRSP slightly affected BP, which was significantly reduced only after 5 weeks of treatment (Table 1). Conversely, much more

TABLE 2. Levels of 24-h proteinuria

Proteinuria (mg/day)	Week 3	Week 4	Week 8
StD-SHRSP	14.1 ± 8.5	14.2 ± 7.9	14.5 ± 3.8
JpD-SHRSP	81.4 ± 82.5*	240 ± 83**	ND
JpD-cap	ND	56.6 ± 26.7***	28.4 ± 11.7
StD-WKY	13.9 ± 1.6	14.1 ± 6	15.9 ± 6.4
JpD-WKY	13.0 ± 2.8	15.8 ± 4.5	ND

Data are mean (mg/day) ± SD. General linear models were applied for statistical analysis. JpD, Japanese high-salt permissive diet; ND, not determined; SHRSP, spontaneously hypertensive stroke prone; StD, standard diet; WKY, Wistar Kyoto.
*$P = 0.0007$ StD-SHRSP vs. JpD-SHRSP (week 3).
**$P < 0.0001$ StD-SHRSP vs. JpD-SHRSP (week 4).
***$P < 0.0001$ JpD-cap vs. JpD-SHRSP (week 4) by post-hoc test with Bonferroni correction.

pronounced was the effect of the drug on the organ damage – that was prevented (Fig. S1, http://links.lww.com/HJH/A906) – and in turn on the proteinuria that, in 5-week treatment, reverted to a normal range (Table 2).

Platelet tissue factor expression

To evaluate the relationship between hypertension and platelet-associated TF, cell-surface and intracellular TF levels were analyzed by whole blood flow cytometry in SHRSP rats as well as in WKY control animals.

The number of platelets expressing TF, both on the cell surface as well as in the cytoplasm, was two-fold higher in hypertensive StD-SHRSP compared with normotensive StD-WKY animals (Fig. 2a and b). Of note, when the samples were analyzed for intracellular expression of TF, about three-fold higher amount of TF-positive platelets was detected compared with that measured with the cell-surface staining.

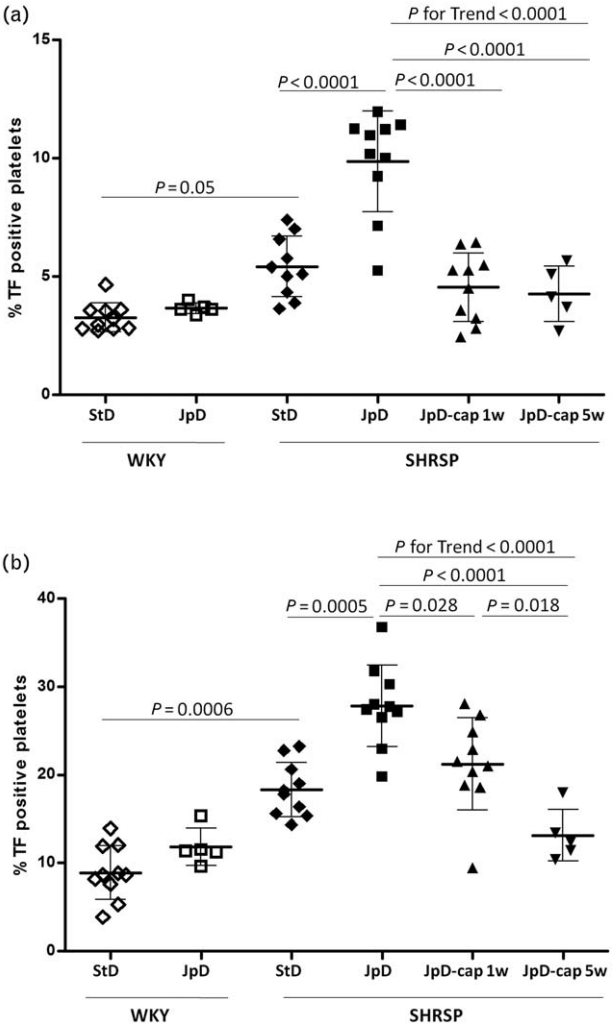

FIGURE 2 Analysis of tissue factor expression in platelets by flow cytometry. Cell-surface (a) and intracellular (b) tissue factor expression was analyzed in platelets, identified as CD61-positive events, of Wistar Kyoto and spontaneously hypertensive stroke prone animals. As no differences were detected within standard diet-Wistar Kyoto and standard diet-spontaneously hypertensive stroke prone animals after 4 and 8 weeks, data were pooled. Data are shown as percentage of tissue factor-positive events. Lines in the dot plots are mean ± SD. General linear models were applied for statistical analysis. Reported *P* values were obtained by post-hoc tests with Bonferroni correction.

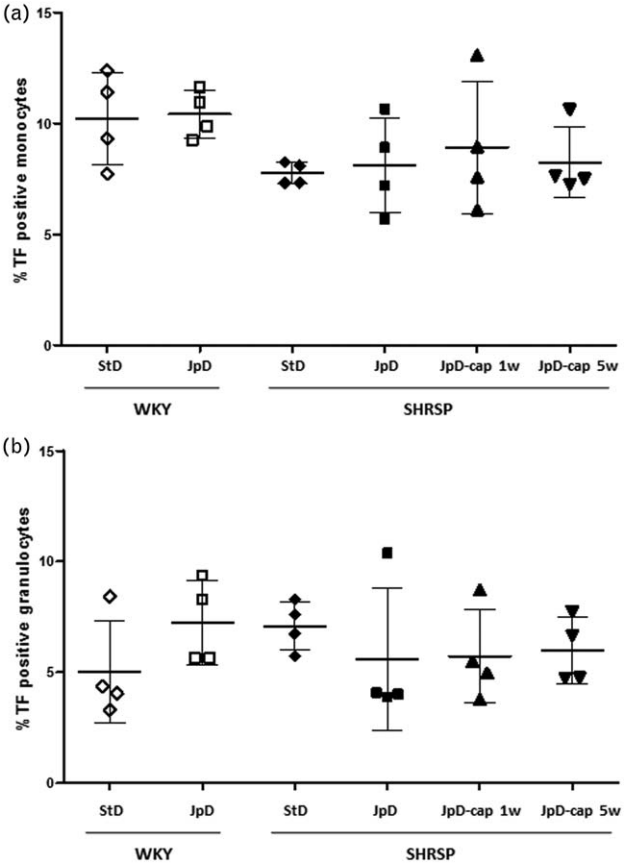

FIGURE 3 Analysis of cell-surface tissue factor expression in monocytes and granulocytes by flow cytometry. Cell-surface tissue factor expression was analyzed in monocytes (a) and granulocytes (b) of a subgroup of Wistar Kyoto and spontaneously hypertensive stroke prone rats. Cells were identified according to their physical properties (high forward and side scatter) and to CD45 expression. Lines in the dot plots are mean ± SD. No significant differences between groups were observed. General linear models were applied for statistical analysis, followed by post-hoc tests with Bonferroni correction.

The percentage of circulating TF-positive platelets further doubled in JpD-SHRSP animals compared with StD-SHRSP, with the onset of a marked organ damage due to the JpD treatment (week 4). Prevention of renal damage by captopril administration reduced the number of TF-positive platelets (week 4), which returned to the StD level after 5 weeks of treatment (Fig. 2a and b).

Flow cytometry analysis of leukocyte TF expression indicated that, in this experimental setting, unlike what was observed for platelets, hypertension did not affect the number of TF-positive monocytes (Fig. 3a) and granulocytes (Fig. 3b), even in the presence of severe organ damage.

Platelet thrombin generation capacity

The CAT assay was performed to assess the thrombin formation capacity of platelets. Platelets from hypertensive StD-SHRSP rats generated thrombin in a faster kinetic rate compared with normotensive StD-WKY (lag time: 16.5 ± 3.1 vs. 22.2 ± 2.1 min, respectively; $P = 0.001$; Fig. S2, http://links.lww.com/HJH/A906), paralleling the

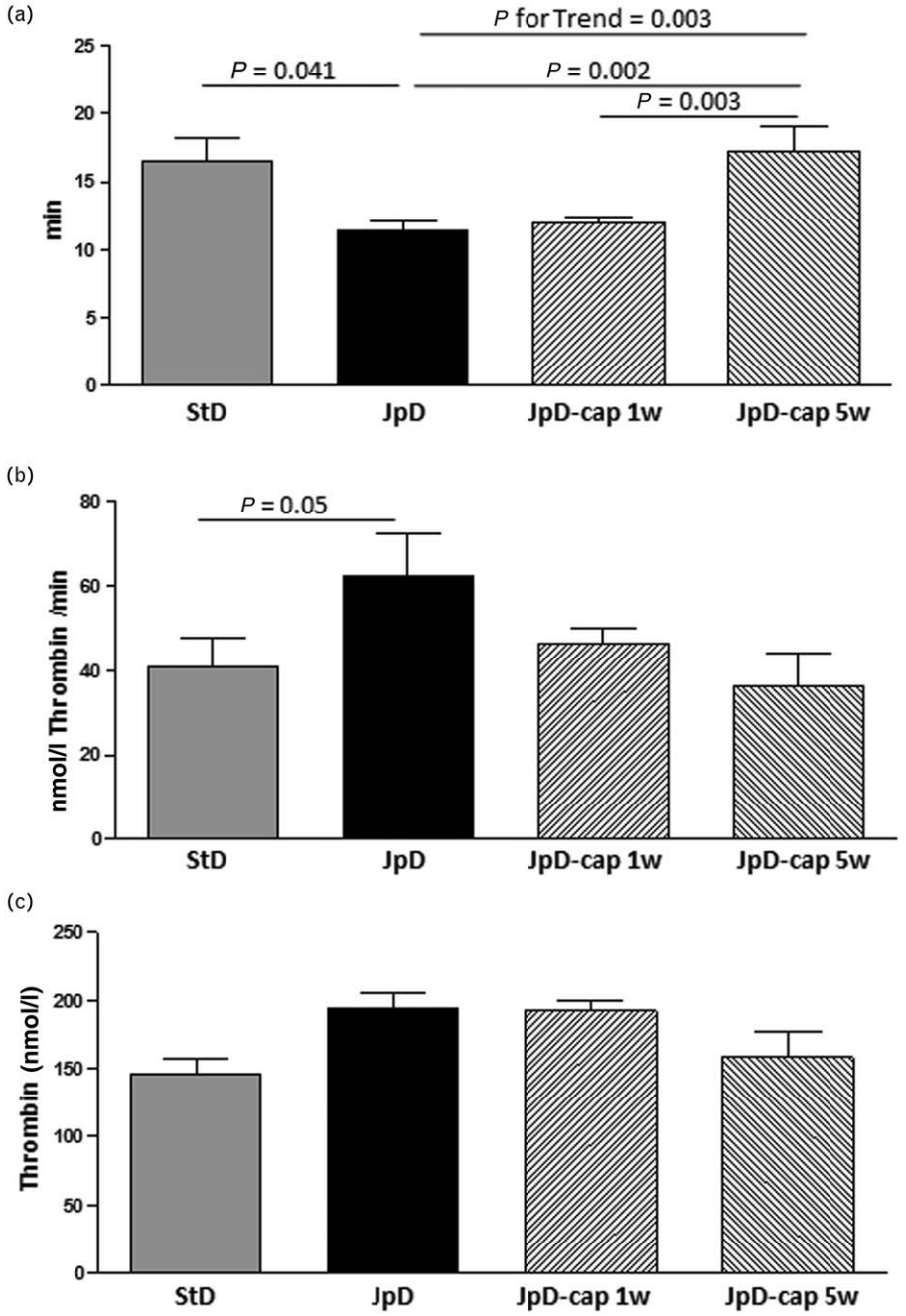

FIGURE 4 Assessment of thrombin generation capacity of platelets. Platelet prothrombotic potential was analyzed by thrombin generation assay (calibrated automated thrombogram) in platelet lysates. Lag time [time needed to start thrombin formation (a)], velocity index [velocity of thrombin formation (b)] and peak height [maximum concentration of generated thrombin (c)] were used as main parameters describing thrombin generation. Representative curves (gray lines) from platelet lysates of standard diet (d) and hypertensive (e) animal are reported. Curves generated in the absence of cell lysates (dashed lines) are shown for comparison. Thrombin generation analyzed in the presence of neutralizing antitissue factor antibody (100 μg/ml AbTF, black lines) is reported. Data in the histograms are mean ± SD. General linear models were applied for statistical analysis. *P* values were obtained by post-hoc tests with Bonferroni correction.

higher percentage of TF-positive platelets circulating in SHRSP animals. The thrombin formation was further faster in JpD-SHRSP compared with StD-SHRSP rats (lag time: 11.4 ± 1.7 vs. 16.5 ± 3.1 min, $P = 0.041$ and velocity index: 62.2 ± 23.0 vs. 40.7 ± 14.2 mmol/l per min, $P = 0.05$, respectively; Fig. 4a and b), and this feature was accompanied by a trend toward a greater amount of thrombin (peak height: 194.3 ± 30.0 vs. 146.0 ± 24.4 nmol/l; $P = 0.09$, Fig. 4c). Of interest, treatment with captopril for 5 weeks was able to restore the thrombin generation capacity of platelets to the values observed in StD-SHRSP animals (Fig. 4a–c), in agreement with the effect on TF expression assessed by flow cytometry.

To investigate the direct contribution of platelet TF to the thrombin generation, the assay was performed after preincubation of cell lysates from StD-SHRSP and JpD-SHRSP rats with a neutralizing anti-TF antibody. In this experimental condition, the onset of thrombin generation was delayed

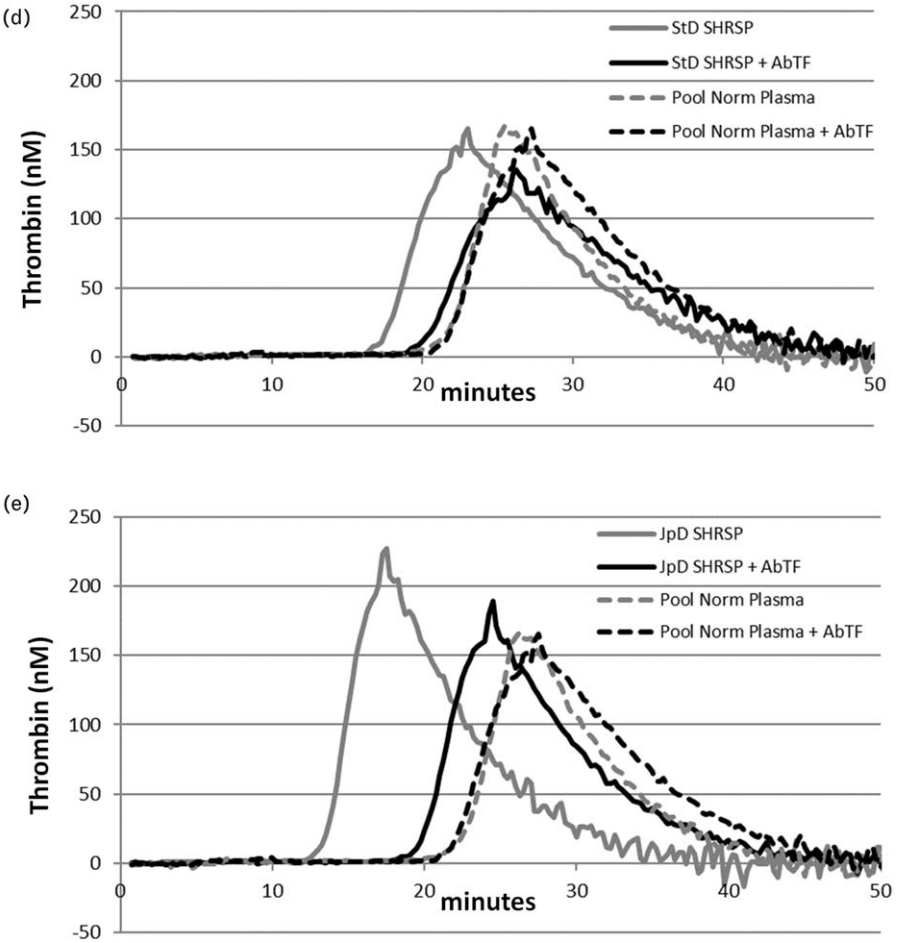

FIGURE 4 (Continued)

becoming similar to that measured in the absence of platelet lysates (i.e. plasma alone; Fig. 4d and e). As expected, the delay in thrombin formation was greater in platelets from JpD-fed animals, reflecting the higher number of TF-positive platelets compared with that of StD-fed rats.

Megakaryocyte tissue factor expression

Bone marrow megakaryocytes were analyzed at sacrifice to verify if the increased number of TF-positive platelets observed in hypertensive SHRSP animals was associated with an increase in TF expression in the platelet progenitor cells. Megakaryocytes were carefully identified taking advantage from imaging flow cytometry according to their physical properties (high forward and side scatter) and high CD61 expression (Fig. S3, http://links.lww.com/HJH/A906 and Fig. 5a). The selected population had a mean cell diameter of $39.2 \pm 6.7\,\mu m$ and showed different degree of polyploidy as assessed by nuclear staining with DRAQ5 (data not shown). Analysis of cell-surface TF expression showed that the number of TF-positive megakaryocytes was significantly higher in hypertensive animals compared with StD-WKY rats (Fig. 5b). It further increased in JpD-SHRSP, in which hypertension is worsened by organ damage. Of interest, the percentage of TF-positive megakaryocytes returned to the StD-SHRSP levels already after 1 week of treatment with captopril (Fig. 5b).

Effect of angiotensin on platelet tissue factor expression

The activity of the renin–angiotensin system is increased in SHRSP after high-salt diet treatment [15]. To evaluate the effect of AngII on platelet activation in terms of surface-associated TF expression, ex-vivo experiments on PRP from normotensive StD-WKY rats were performed. Platelet stimulation with a classical agonist, such as collagen (1 and $4\,\mu g/ml$), was analyzed as positive control (Fig. 6a) and resulted in a concentration-dependent increase in the number of surface-associated TF-positive platelets. Stimulation with Val5AngII resulted in a concentration-dependent increase in the number of TF-positive platelets compared with untreated cells. Preincubation of platelets with valsartan, a selective AT1 antagonist, inhibited TF upregulation induced by AngII (Fig. 6b).

The effect of AngII on TF expression was evaluated also in human platelets isolated from healthy individuals. Stimulation of platelets with Val5AngII resulted in a concentration-dependent increase of the number of TF-positive platelets (Fig. 6c), paralleling results obtained in animals.

DISCUSSION

The current study shows for the first time that the number of TF-positive megakaryocytes and in turn the number of

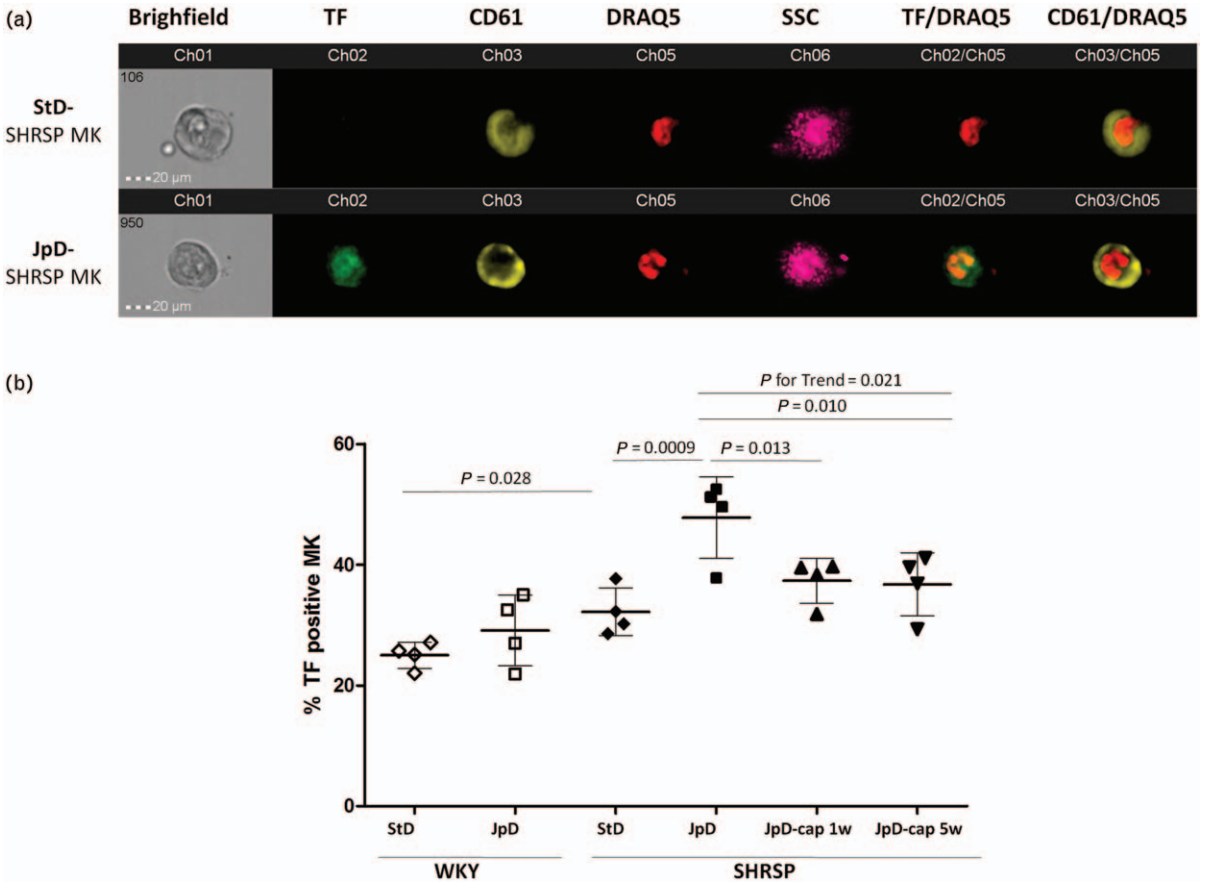

FIGURE 5 Analysis of cell-surface tissue factor expression in megakaryocytes by flow cytometry. Bone marrow megakaryocytes were identified by imaging flow cytometry according to their physical properties and high CD61 expression. Cell nuclei were stained with DRAQ5 dye. Representative compensated images of standard diet (a1) and Japanese high-salt permissive diet (a2) spontaneously hypertensive stroke prone megakaryocytes are reported in panel (a). Cell-surface tissue factor expression in megakaryocytes from Wistar Kyoto and spontaneously hypertensive stroke prone rats was quantified by traditional flow cytometry and reported as percentage of tissue factor-positive cells (b). Lines in the dot plots are mean ± SD. General linear models were applied for statistical analysis. *P* values were obtained by post-hoc tests with Bonferroni correction.

circulating platelets carrying a functionally active TF is significantly increased in hypertensive compared with normotensive status. This leads to a higher platelet prothrombotic potential as evidenced by a faster and greater thrombin generation capacity, which is blunted by a specific anti-TF antibody. This picture is further exacerbated when hypertension is complicated by end-organ damage and endothelial cell dysfunction, which however can be reverted by treatment with the angiotensin-converting enzyme (ACE) inhibitor captopril.

Studies carried out in the past 15 years have documented that TF, the transmembrane glycoprotein key activator of the blood coagulation cascade and of thrombus formation, is expressed not only by activated endothelial cells or leukocytes, but also by circulating platelets of human, rat and pig origin [24]. This finding has placed in a different perspective the involvement of platelets in the thrombin generation process, being their contribution not only in the propagation of the clotting cascade, in which they provide the negatively charged phospholipid bilayer for the assembly of the coagulation factors, but also in the initiation phase, being TF the trigger of the coagulation cascade. TF is stored in the platelet cytoplasm and, upon activation with classical agonists such as thrombin or ADP, it is rapidly translocated on the cell surface in which it can bind factor VIIa thereby leading to thrombin generation [5,25].

Platelets are generated by bone marrow resident megakaryocytes, a quite rare cell population, accounting for less than 0.01% of the total cells present in the bone marrow, which constantly release new platelets into the bloodstream. We have recently shown that functionally active TF is present in human megakaryoblasts and its expression increases in megakaryocytes which then transfer it to a subset of shed platelets in which it contributes to clot formation. Although platelets do not have a nucleus, they also contain, in addition to the protein, megakaryocyte-derived TF pre-mRNA and mRNA that can be used, upon activation, for de novo protein synthesis [6]. It is worth mentioning, in this regard, that the TF mRNA species are mostly stored in platelets containing a negligible amount of TF protein. These TF mRNA-containing platelets can therefore become TF protein-containing platelets upon activation, as just mentioned above.

Of interest, the number of TF-positive platelets has been previously shown to significantly increase in several pathological conditions, including diabetes and acute coronary syndromes, compared with healthy status, providing an additional explanation of the higher thrombogenicity

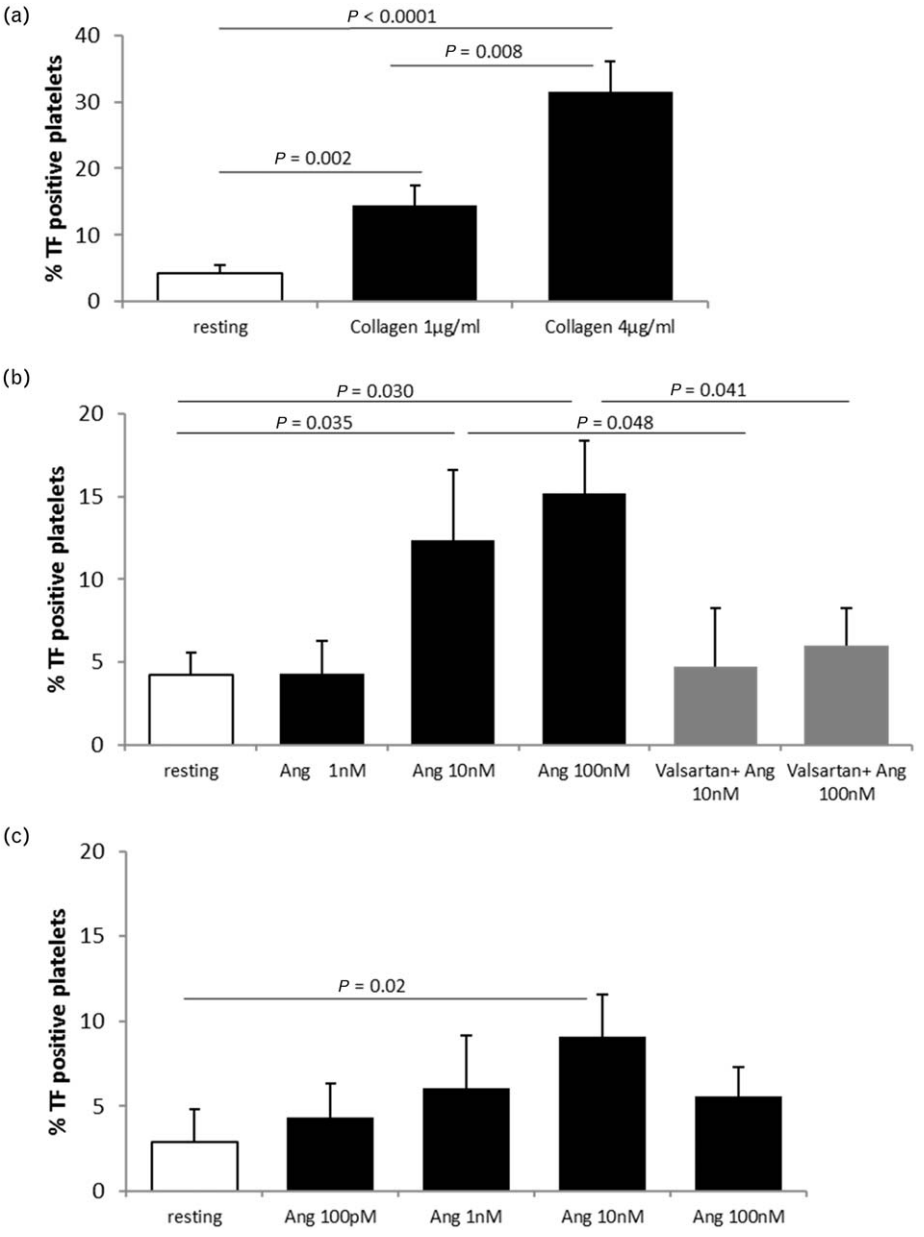

FIGURE 6 Flow cytometry analysis of cell-surface tissue factor expression in rat and human platelets stimulated with angiotensin II. Platelet-rich plasma from normotensive Wistar Kyoto rats was stimulated with increasing concentration of collagen (a) or, a selective AT1 agonist (b), and stained with polyclonal antitissue factor antibody labeled with Alexa Fluor 633. The effect of AngII on human platelets is also reported (c). Mean percentage of tissue factor-positive cells ± SD, in each experimental setting, is reported as histogram. ANOVA was applied for statistical analysis. *P* values were obtained by post-hoc test.

documented in these clinical settings [9,10]. The information so far accumulated on the regulation of platelet TF do not allow us to understand whether this increase is due to mechanisms taking place in the bloodstream, ultimately resulting in the translation of the platelet TF mRNAs, or in the bone marrow, in which megakaryocytes could increase the release of TF-positive platelets into the circulation. As one can easily understand, this is an issue quite hard to be solved in humans. Indeed, as far as the megakaryocytes are concerned, their location being hardly accessible, together with their low amount, definitely limits the possibility to perform studies on mature megakaryocytes in humans.

Conversely, taking advantage from the SHRSP animal model, we were able to gain further insights into these mechanisms assessing TF expression in megakaryocytes by flow cytometry. The data here reported clearly show that hypertension is associated with a significant increase of the number of TF-positive megakaryocytes, which in turn may shed more TF-positive platelets in the circulation. In a severe hypertensive status, such as that induced by the administration of the high-salt diet and which is characterized by a severe renal impairment and endothelial dysfunction, the number of TF-positive megakaryocytes further increases.

As the presence of TF in megakaryocytes has been only recently characterized, data on its transcriptional regulation in these cells are so far completely missing and will be matter of future investigations. At present therefore we can

only try to interpret our data borrowing the knowledge on TF regulation learnt with other cells. The inducible expression of TF, which we believe is taking place in megakaryocytes in our experimental setting, is under the control of the transcription factor NF-κB. This is activated, among the others, by reactive oxygen species, inflammatory stimuli, AngII (the effector arm of the renin–angiotensin system), all key players in the vascular complications of hypertension [26–28]. Indeed, consistent data in the literature have shown that both inflammatory stimuli and AngII stimulate *TF* gene transcription and expression in human monocytes, rat aortic endothelial cells and vascular smooth muscle cells [29–33]. As both inflammation and renin–angiotensin system are increased in hypertensive SHRSP rats, especially when end-organ damage and endothelial dysfunction are also present [9,14,15,34,35], it is worth to speculate that they might affect the *TF* gene transcription in megakaryocytes as well, but, of course, this hypothesis has to be proved in future investigations.

AngII is also able to activate platelets, increasing cytosolic-free calcium concentration and promoting both spontaneous and agonist-induced aggregation [35]. Thus, we tested in ex-vivo experiments its effect on platelet TF expression. Of interest, we found that stimulation of platelets from normotensive rats with a Val5AngII resulted in a rapid, concentration-dependent increase of cell-surface TF expression. This effect was completely abolished by preincubating platelets with valsartan, a selective AT1 antagonist, thus proving the specificity of the mechanism involved. Of note, similar results were obtained also with human platelets, suggesting that the same regulation may take place also in humans.

As previously mentioned, however, prolonged platelet activation results in mRNA translation and de novo protein synthesis. As the activated renin–angiotensin system is more pronounced in hypertension [36], platelets may be 'chronically' exposed to AngII. We cannot therefore rule out the possibility that AngII may also exert its effect on platelet TF expression through a mechanism acting at the mRNA levels.

In the current study, we provided the evidence that, when organ damage [37] and BP are controlled by captopril treatment, the number of TF-positive megakaryocytes returns to the basal levels, thus restoring also the number of TF-positive platelets. As this effect was observed already after 1 week of treatment, it is tempting to speculate that a clear anti-inflammatory and antithrombotic properties of the pharmacological renin–angiotensin system blockade, above and beyond its antihypertensive properties, is taking place. Indeed, it has been previously reported, that the pharmacological inhibition of the renin–angiotensin system by means of drugs such as ACE inhibitors or AT1 blockers were able to modulate TF in different cell types (reviewed in Celi *et al.* [2]). We previously showed the capacity of captopril to downregulate endotoxin-induced TF expression and activity in human monocytes [38]. Valsartan was shown to reduce TF expression in vascular cells such as endothelium, smooth muscle cells and adventitia cells in transgenic rat overexpressing human renin and angiotensinogen genes [39].

Of note, this is not the first study suggesting the potential involvement of platelet TF to thrombotic events. Our data are indeed in line with a recent study by Tyagi *et al.* [12] who reported, using a proteomic approach, that the exposure of rats to a hypoxic stimulus results in the upregulation of platelet TF expression and in the processing of the TF pre-mRNA into mature RNA.

The contribution of TF to the prothrombotic phenotype described in hypertension has been mainly linked to the expression of the protein by activated monocytes and endothelial cells. Nevertheless, data accumulated so far on the characterization of platelet TF expression in healthy as well as in pathological conditions indicate that the time has come to consider activated platelets as a considerable source of functionally active TF. In this regard, we have previously reported that in patients with acute coronary syndrome, although on a per-cell basis monocytes carry more TF protein than platelets, the total amount of TF carried by the platelets present in 1-μl blood volume far exceed (1000-fold difference) the amount carried by monocytes [28]. Finally, TF could be transferred from activated platelets to monocytes and neutrophils through microvesicles, that are a considerable source of functionally active TF and have a high procoagulant activity [40].

In our experimental setting, the hypertension did not upregulate TF in monocytes and granulocytes. It is worth mentioning in this regard that data reported in literature are quite controversial. Although some studies clearly show that AngII is able to increase in-vitro monocyte-TF expression and activity [29,30], others suggest that hypertensive patients without any other cardiovascular risk factors have levels of monocyte-associated TF comparable to that of normotensive controls. The presence of additional risk factors, such as carotid atherosclerosis, together with hypertension may significantly influence TF levels in these cells [41].

In summary, our data suggest that new players, the TF-expressing megakaryocytes and platelets, might contribute to the well documented prothrombotic state observed in the onset and evolution of a hypertensive status.

In addition, although the effect of the other players involved in the end-organ damage and endothelial dysfunction could not be excluded, the renin–angiotensin system may contribute to the procoagulant phenotype reported in hypertensive animals. By ex-vivo experiments, we indeed provided the direct evidence that AngII, through an AT1-specific mechanism, induces the TF exposure on the platelet membrane, and this effect was observed also in human platelets. Although data obtained with the animal model need to be confirmed also in the clinical setting, it could be anticipated that drugs interfering with the renin–angiotensin system, in addition to lowering BP, may be beneficial for preventing the increased thrombogenicity observed in patients with hypertension.

Limitation of the study

The experimental setting used in the current study does not allow to ascertain whether the modulation of TF expression in megakaryocytes and platelets exerted by captopril is related to changes in the renin–angiotensin system or to the protective effect on the end-organ damage observed in severe hypertensive animals. Indeed, inflammatory processes as well as endothelial dysfunction taking place in the SHRSP animal model may also contribute, in addition to the renin–angiotensin

system activation, to the prothrombotic phenotype of megakaryocytes and, in turn, of circulating platelets. Further studies on animals treated with drugs able to reduce BP without impacting AngII levels and/or the extent of target organ damage will help to unravel this issue.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

Strengths: The authors perform in vivo and in vitro assessments to demonstrate that angiotensin II upregulates tissue factor expression uniquely in platelets.
Weaknesses: Ultimately, the authors will need to determine the relationship between hypertension and/or target organ damage and tissue factor expression.

Reviewer 2

The study by Brambilla *et al.* examined the effect of captopril on cell-associated tissue factor (a key activator of coagulation and thrombus formation) expression in SHR-stroke prone rats fed either a standard of Japanese high-salt permissive diet (i.e. altered sodium/potassium ratio, low protein content, and 1% NaCl supplement in the drinking water. SHR-stroke prone rats fed either a normal or high-salt diet were found to have increased platelet tissue factor expression in megakaryocytes) as compared to WKY control rats. A high-salt diet exacerbated tissue factor expression in SHR-stroke prone rats and was associated with a greater degree of hypertension and renal injury compared to WKY control and SHR-stroke prone rats fed a standard diet. A role for the rennin–angiotensin system was implicated in these responses, as captopril (an angiotensin-converting enzyme inhibitor) was associated with a reduction in the same end-points, including thrombin generation assays. These findings demonstrate an association between tissue factor expression in a genetic model of hypertension and in response to a high-salt diet. The observed increase in tissue factor in platelets may promote, in part, an increased risk of thrombotic events, such as myocardial infarction, ischemic stroke, and renal injury, commonly observed in hypertensive patients.