Evidence From Comparative Investigations That Impaired Platelet Activation Is Not Specific for Stroke-Prone Spontaneously Hypertensive Rats

Andreas Klee, PhD; Sylvia Vater; Geert W. Schmid-Schönbein, PhD; Dirk Seifge, DVM, PhD

Background and Purpose: Platelet behavior of Sprague Dawley (SD), Wistar (WI), Wistar-Kyoto (WKY), spontaneously hypertensive (SHR), and stroke-prone spontaneously hypertensive rats (SHRSP) was studied in vivo to evaluate the importance of hypertension-related hemostatic disorders.

Methods: The study was based on the model of stimulus-induced pulmonary microembolization of labeled platelets. After injection of 51Cr-labeled homologous platelets into urethane-anesthetized rats, the organ distribution of the platelets was continuously monitored by gamma detectors. Count rates of two detectors—one placed above the animals’ thoraxes (C1), the other above their abdomens (C2) —and the ratio of C1:C2 were calculated. The following platelet activators were applied intravenously: adenosine diphosphate (ADP; 50 µg/kg), collagen (100 µg/kg), and thrombin (50 IU/kg).

Results: All three substances caused a reversible pulmonary accumulation of the labeled platelets and hence an increase in C1/C2 (AC1/C2%). ADP induced a shift of 75% in SD, 52% in WI, 32% in WKY, 30% in SHR, and 31% in SHRSP. Thrombin-mediated shift was 79% in SD, 64% in WI, 58% in WKY, 48% in SHR, and 54% in SHRSP. Collagen induced a shift of 85% in SD, 96% in WI, 84% in WKY, 56% in SHR, and 62% in SHRSP.

Conclusions: Because indistinguishable results were observed in both hypertensive strains, we conclude that impaired platelet aggregation is not specific for SHRSP. Hence, it may not primarily be responsible for the increased occurrence of stroke in these animals. (Stroke. 1993;24:1528-1533.)

Key Words • hemostasis • platelet activation • rats

Under physiological conditions hemostasis is a carefully regulated system maintaining the integrity of the circulation. Disturbances of this system will lead to hemostatic activation. Under pathophysiological conditions enhanced activity of its components can be observed, for example, hyperaggregability of platelets. An increased activation of platelets implies their intravascular consumption. In this connection diseases such as myocardial infarction, thrombosis, formation of embolisms, or disseminated intravascular coagulation may occur.

Arterial hypertension is assumed to be an important risk factor for thrombotic diseases. A high incidence of thrombosis in coronary and cerebral arteries observed in patients with hypertension may indicate an enhancement of platelet function in these patients. Enhanced adhesion and aggregation as well as hypoaggregateability of platelets from hypertensive patients have been reported. The inconsistency of these results is thought to be due to varying experimental conditions and heterogeneity of the patient groups.

Inbred spontaneously hypertensive rats (SHR) and spontaneously hypertensive stroke-prone rats (SHRSP), a substrain of SHR, established in 1963 and 1974, respectively, by Okamoto et al., are considered to be animal models related very closely to human hypertension. These animals offer the advantage of investigating hypertension-related alteration in platelet function under controlled and comparable regimens. Activation of platelets of these rat strains has been examined by different authors with inconsistent results regarding hypo- or hyperaggregability. Because most of these results have been obtained from in vitro experiments, different methodology (washed platelet or platelets in plasma, different anticoagulants) may at least partially explain this discrepancy.

It was the aim of our study to describe platelet function of SHRSP by comparing the intravascular reactivity of SHR and SHRSP platelets with platelets in Sprague Dawley (SD), Wistar (WI), and Wistar-Kyoto (WKY) rats. Labeling platelets with 51Cr allows the study of the behavior of platelets continuously in vivo by noninvasive methods. This model has recently been described extensively for the SD strain. 17

Materials and Methods

Chemicals

Chemicals were obtained from the following companies: prostaglandin E1 (stored in ethanol at −70°C) and thrombin (rat) (dissolved in phosphate buffered saline)
from Sigma Chemical Co, Deisenhofen, Germany; urethane from Riedel-de-Haen, Hannover, Germany; heparin from Novo Industries, Mainz, Germany; ADP from Boehringer, Mannheim, Germany; type I collagen (equine) from Hormonchemie, Munich, Germany; and $^{51}$Cr as Na$_2$CrO$_4$ in saline from Du Pont de Nemours (NEN), Dreieich, Germany.

**Animals**

Male SD, WI, WKY, SHR, and SHRSP, 330 to 380 g body weight, were used for the in vivo studies. They also served as donors of homologous platelets. All animals were obtained from Moltegaard, Skensved, Denmark, at the age of 16 to 18 weeks.

**Isolation and Radiolabeling of the Platelets**

Cardiac puncture under ether anesthesia was performed to obtain heparinized rat blood (50 IU/mL). After centrifugation (200g; 10 minutes) the platelet-rich plasma was diluted four-fold with cold-free Tyrode's solution (CFTPG) containing 300 ng/mL prostaglandin E$_1$. Platelets were pelleted by centrifugation at 750g (10 minutes). The supernatant was discarded, and the platelets were resuspended in CFTPG. The platelets (18x10$^9$/mL) were incubated with 200 $\mu$Ci $^{51}$Cr(VI)-sodium chromate for 5 minutes at 37°C and washed twice by centrifugation at 750g for 5 minutes.

**Preparation of the Animals**

Arterial blood pressure was measured before the experiments using the tail cuff method. Rats were anesthetized under light ether using urethane (1.25 g/kg IM) and were allowed to breathe spontaneously. The femoral artery, femoral vein, and carotid artery were catheterized.

**Platelet Activation In Vivo**

$^{51}$Cr-labeled platelets were administered into the femoral vein. Platelets were allowed to circulate after injection at least 60 minutes before their activation was triggered. The labeled platelets were continuously monitored in the thoracic (C1) and abdominal region (C2) using collimated sodium crystal scintillation detectors. Counts from up to four animals were collected simultaneously by means of an eight-channel spectrometer and were evaluated by a microcomputer (AIMS 8000, Mumed Ltd, London, UK). Counts were calculated as the ratio C1:C2 and as percent increase over the count ratio at 20-second control periods before the bolus injection of platelet-activating drugs (ADP, collagen, or thrombin). During the experiments heart rate and blood pressure were recorded by the carotid artery catheter.

**Platelet Aggregation In Vitro**

Heparinized platelet-rich plasma, obtained as described above, was adjusted to 250,000 platelets per microliter using homologous plasma. Platelet activation was studied photometrically in a four-channel aggregometer. Additionally, aggregability of $^{51}$Cr-labeled platelets was investigated after washing the platelets with CFTPG and resuspending them in heparinized rat plasma (50 IU/mL).

### Table 1. Arterial Platelet Count and Arterial Blood Pressure in SD, WI, WKY, SHR, and SHRSP

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Platelets, $\times 10^9$/µL</th>
<th>Awake $P_a$ mm Hg</th>
<th>Anesthetized $P_a$ mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>909/17</td>
<td>154/7</td>
<td>98/12</td>
</tr>
<tr>
<td>WI</td>
<td>833/29</td>
<td>150/7</td>
<td>92/8</td>
</tr>
<tr>
<td>WKY</td>
<td>796/9</td>
<td>135/2</td>
<td>83/7</td>
</tr>
<tr>
<td>SHR</td>
<td>653/20*</td>
<td>226/6*</td>
<td>117/5</td>
</tr>
<tr>
<td>SHRSP</td>
<td>633/12*</td>
<td>225/6*</td>
<td>125/17*</td>
</tr>
</tbody>
</table>

$P_a$, mean arterial pressure; SD, Sprague Dawley; WI, Wistar; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive; SHRSP, stroke-prone spontaneously hypertensive.

Values are mean±SEM.

*Significant vs WI ($P<.05$).

**Platelet Counting**

Blood was taken via the femoral artery and heparinized (50 IU/mL). After fourfold dilution with CFTPG and sedimentation of the erythrocytes and leukocytes, platelet count in the supernatant was determined using an electronic cell counter (Coulter Counter, Coulter Electric, Krefeld, Germany).

**Statistics**

Time-dependent shifts of C1:C2, caused by the injection of platelet-activating drugs, were expressed as $\Delta$C1:C2 (%). $\Delta$C1:C2, mean arterial pressure, and heart rate were expressed as the mean±SEM. Unless otherwise indicated, 10 rats were used in each experiment. Results were compared using Student's $t$ test and Bonferroni corrected $t$ test when multiple comparison was made. Values of $P<0.05$ indicated statistical significance. The same probability values were used to describe linear correlations.

**Results**

**Preparation of Labeled Platelets and Recovery**

The purification and labeling of the platelets of the hypertensive substrains of WKY required a larger amount of blood obtained by cardiac puncture compared with SD. This was due to decreased platelet counts in blood of SHR and SHRSP, because blood gained by cardiac puncture or by catheterization of the femoral artery contained significantly lower platelet counts (Table 1). Despite the weak cardiovascular side effects of urethane,18 the hypertensive rat strains reacted very sensitively at anesthesia. Mean arterial blood pressure was considerably decreased (Table 1). No strain-specific differences in heart rates could be detected; heart rates varied from 200 to 330 beats per minute (data not shown).

Rats of each strain were injected with 20 to 24 $\mu$Ci $^{51}$Cr bound to $4.5x10^9$ platelets. Erythrocytes and leukocytes accounted for less than 1% of the cell suspension. After injection the labeled platelets rapidly distributed in the circulation. Thoracic and abdominal radioactivity recorded by detectors C1 and C2 stabilized within 3 minutes. Recovery of the platelets in blood measured 60 minutes after injection was higher than 85% and did not vary markedly among the different
TABLE 2. Aggregation of WI and SHRSP Platelets In Vitro

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Freshly Prepared Platelets</th>
<th>51Cr-Labeled Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI</td>
<td>71%</td>
<td>56%</td>
</tr>
<tr>
<td>SHRSP</td>
<td>52%</td>
<td>42%</td>
</tr>
</tbody>
</table>

WI, Wistar; SHRSP, stroke-prone spontaneously hypertensive. Platelets were resuspended in homologous plasma and adjusted to 250 000 per microliter. Values represent the mean of four individual experiments. Platelets obtained from three rats were pooled.

Platelet Aggregability

In vitro aggregation of freshly prepared platelets, studied by adding ADP (10^{-6} M) to platelet-rich plasma, was compared with the aggregation of 51Cr-labeled platelets. 51Cr-labeled WI and SHRSP platelets, which were prepared in the absence of prostaglandin E1, showed a decrease in aggregability (Table 2).

Activation of Labeled Platelets In Vivo

51Cr-labeled platelets were stimulated with one of the activators (50 µg/kg ADP, 100 µg/kg collagen, or 50 IU/kg thrombin). Intravenous injection of ADP induced a reversible thoracic platelet accumulation resulting in augmented counts of C1 and a concomitant decrease in C2 (Fig 1). This reaction corresponded to an increase in the ratio C1:C2. The effect of ADP was dose dependent. By means of a double-reciprocal plot, the sigmoidal dose-response curve could be transformed having a linear correlation between 1/dose and C2:C1. Based on this curve, a maximal shift of C1:C2 of 76% was calculated.

Dosages of ADP and the other activators causing a submaximal shift of C1:C2 in SD were taken to investigate strain-specific platelet behavior.

Pulmonary platelet accumulation described as agonist-induced maximal shift in the ratio C1:C2 depended critically on the choice of activator (Fig 2). Compared with SD, all WI substrains exhibited a reduced reaction after injection of ADP and thrombin. This effect was most obvious for ADP in WKY and its hypertensive substrains, whereas the platelets' response to thrombin was only slightly weakened in the WI substrains. Injection of collagen, however, resulted in a pulmonary

FIG 1. Time-dependent effect of an intravenous injection of ADP (50 µg/kg) on platelet-bound pulmonary radioactivity (C1) and abdominal radioactivity (C2). Double-reciprocal plot was calculated from the dose-dependent maximal increase in the ratio C1:C2 (DC1/C2). Values are the mean of 10 experiments.

FIG 2. Pulmonary platelet accumulation induced by intravenous injection of ADP (50 µg/kg), thrombin (50 IU/kg), and collagen (100 µg/kg) in different rat strains. Values were calculated as the maximal increase in the ratio C1:C2 (DC1/C2) and represent the mean ± SEM of 10 experiments. * indicates significant difference (P<.05).
platelet accumulation decreasing stepwise in the inbred substrains of WI.

Simultaneously recorded mean arterial blood pressure and heart rate of WI and SHRSP fell reversibly after injection of ADP and collagen (Fig 3). In both rat strains ADP-induced hypotension and bradycardia occurred rapidly and recovered faster in comparison to collagen. A diminished accumulation of labeled platelets in SHRSP accompanied a less pronounced cardiovascular alteration. The hypertensive rat strains had an enhanced preexperimental mean arterial blood pressure, but this enhancement was not correlated with the extent or duration of the pulmonary platelet accumulation. We observed only a weak correlation between maximal rise in \( \Delta C1:C2 \) and cardiovascular alteration in WI and SHRSP (correlation coefficient for \( \Delta C1:C2 \) vs arterial pressure: \( r = -0.72 \) [WI], \( r = -0.57 \) [SHRSP]; correlation coefficient for \( \Delta C1:C2 \) vs heart rate: \( r = -0.51 \) [WI], \( r = -0.46 \) [SHRSP]). The correlation was found to be significant for \( \Delta C1:C2 \) versus arterial pressure in WI.

**Discussion**

Modern management of acute stroke patients has included various attempts to salvage damaged but viable tissue in or around the vascular brain lesion. Controlled trials, however, have mostly been negative. Trials of antiplatelet treatment with beneficial effects and pathological studies indicate that platelets play an important role in the pathogenesis of cerebral ischemic syndromes, because a substantial number of attacks may arise from platelet-fibrin emboli that originate from lesions in the carotid arteries or from the heart.

Our experiments were conducted to describe platelet function of SHRSP in comparison with related strains to investigate the importance of altered platelet function for stroke in SHRSP. The SD strain was used in the study to describe strain- and substrain-specific variations. Platelets of the WI strain were less sensitive when activated by ADP and thrombin. An enhanced collagen-induced platelet activation in WI is gradually lost by breeding WKY and the hypertensive rat substrains. Strain-specific variations in platelet aggregation were also observed by Dwyer and Meyers.

The in vivo response of WKY, SHR, and SHRSP platelets to ADP was decreased very similarly in comparison to WI. Whereas ADP is believed to play a key role in the thrombogenesis in SD, this activator is less effective on SHR and SHRSP platelets. The discrepancy between our findings of a weakened platelet reactivity in all three WI substrains, in contrast to an effect confined to the hypertensive rats, is presumably due to problems of in vitro experiments. The use of washed platelets, the concentrations of calcium and fibrinogen, and the anticoagulant used for the preparation of platelet-rich plasma affect platelet aggregation in vitro. Because our assay does not require anticoagulants having physiological calcium and fibrinogen concentrations, the results probably show more accurate information about platelet reactivity in vivo.
Thrombin induced platelet activation in WI that was not significantly different from the WI inbred substrains. An impaired calcium-dependent response of the SHR and SHRSP platelets after injection of ADP suggests that mechanisms other than a greater rate of agonist-induced uptake of calcium are involved in platelet activation by thrombin.25 In contrast to other authors,27 Huzzoor-Akbar and Anwer28 found that platelets of hypertensive rats exerted a strong response in vitro when stimulated with thrombin.

In accordance with our observations, Yamori et al29 described an attenuated aggregation of SHRSP platelets caused by heterologous collagen. Because of its altered chemical structure,30 however, vascular collagen of SHRSP may account for a significantly stronger mural thrombus formation induced by laser injury compared with WKY.31

At an advanced age SHRSP were found to exhibit increased coagulative and decreased fibrinolytic activities in blood vessels, which is consistent with necrotic alterations in the intima that lead to a sustained activation of platelets with degranulation.32 Beside its vasoactive effect, endothelium-derived relaxing factor inhibits platelet aggregation and adhesion to the vessel wall.33 Because vasodilatation is impaired in SHR and SHRSP, probably in part mediated by decreased release of endothelium-derived relaxing factor,34 this factor could account for a decreased platelet aggregation resulting from exhaustion of preactivated platelets. Accordingly, an agonist-specific primary platelet dysfunction as well as an exhaustion due to permanent activation could account for the intravascular reaction of labeled platelets. Platelet numbers differing from those of normotensive rats were observed by us and others.35 They are only partially caused by the consumption of platelets due to vascular lesions, because these changes occur at early hypertensive stages before the appearance of these lesions.36

It remains to be mentioned that the response of labeled SHR and SHRSP platelets to the agonist was probably unrelated to an elevated basal mean arterial pressure, because no correlation between this parameter and the extent of platelet accumulation could be detected. To evaluate the effect of the labeling procedure on the strain-specific pulmonary platelet accumulation, we measured ADP-induced aggregation of WI and SHRSP platelets in freshly prepared suspensions and after labeling with 51Cr. We found a decrease of reactivity in both strains. This effect was thought to be due to the absence of the platelet-protective prostaglandin E1. This metabolite is well suited to prepare platelets for studying pulmonary platelet accumulation because it is rapidly eliminated from the circulation. Its platelet-inhibiting activity, however, disturbs platelet aggregation in vitro. Likewise the reduced aggregability of SHRSP platelets in vitro before and after labeling, as well as aggregation in vivo, makes a SHRSP-specific preactivation and exhaustion during platelet preparation very unlikely. Furthermore, the platelets’ response was unrelated to a decreased platelet count, since the same amount of labeled platelets was injected into all animals. Despite a greater shift in ΔCl−:Ca2+ after injection of collagen compared with ADP, both activators produced a similar fall in mean arterial pressure. Because ADP is known to release endothelium-derived relaxing factor,36 an endothelium-dependent vasodilatation, as well as a reflexive hypotension resulting from the pulmonary platelet embolization, may contribute to the hypotensive responses.

The observations that WKY, SHR, and SHRSP platelets exhibited the same weakened sensitivity against ADP, the response of SHR and SHRSP platelets to collagen was decreased very similarly, an effect also appearing in WKY; and SHR death is mostly caused by cardiovascular decompensation, whereas SHRSP die mainly from cerebral hemorrhage and infarction24 suggest that, despite altered platelet aggregation, additional factors account for the incidence of stroke in SHRSP. Impaired platelet aggregation as well as reduced platelet counts may enhance the bleeding risk and hence contribute to susceptibility to hemorrhagic strokes. Another possibility of platelets indirectly increasing the risk of stroke-related disorders has to be considered. Platelets are known to produce mediators that could interact with the circulating leukocytes, especially the granulocytes.37 These cells were found to be elevated and spontaneously activated in SHR, increasing with the animals’ age.38 On the other hand, we were able to demonstrate an aggregation of rat platelets mediated by activated granulocytes.39 Accordingly, a mutual activation of platelets and granulocytes together with an impaired endothelial function might contribute to an enhanced risk for vascular injury in SHRSP.

We conclude that a platelet reactivity depending strongly on the experimental design described above offers the opportunity to study special aspects of an altered platelet function. However, it makes it more difficult to evaluate antiplatelet drugs in the primary or secondary prevention of stroke in patients. According to our experiments, comparative investigations with SHR or SHRSP platelets should also include WI platelets.

Acknowledgments

We wish to thank Hoechst AG, Werk Kalle-Albert for supporting the study and providing the technical equipment.

References

10. Huzzoor-Akbar A, Anwer K. Evidence that the rat is not an appropriate model to study the role of prostaglandins in normal or abnormal platelet aggregation. Thromb Res. 1986;41:555-566.
pulmonary platelet aggregation in wistar rats


The convergence of two lines of research makes the study by Klee et al especially topical. First, a hot area of research in vascular biology is the interaction between platelets and endothelium.1 If there is alteration of platelets, as well as endothelium by hypertension, this will have major implications for understanding pathophysiology of transient ischemic attacks2 and perhaps stroke. Second, several new antiplatelet and antithrombotic agents are under intense study. The mechanisms of action of some of the new agents are fundamentally different from aspirin. Because effective, novel antiplatelet agents are likely to become available in the next few years, it is critical to define the role of platelets in the cerebrovascular complications of hypertension.

This is a challenging area of research. Previous studies of platelet activation in vitro have attempted to determine whether platelets are abnormal in hypertensive patients and experimental animals. The new approach in the study by Klee et al was to activate platelets in vivo in an experimental model of spontaneous hypertension. It is difficult to clarify the relative importance of multiple factors that influence activation of platelets in vivo, including endothelium, leukocytes, and hormones, and to clarify ways in which these factors are altered by hypertension. But the reward for a better understanding of mechanisms that affect platelets, and their actions in vivo, is likely to be improved prevention, and perhaps therapy, in cerebral vascular complications of hypertension.

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References
Evidence from comparative investigations that impaired platelet activation is not specific for stroke-prone spontaneously hypertensive rats.
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Stroke. 1993;24:1528-1533
doi: 10.1161/01.STR.24.10.1528

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