Hypoaggregability of Washed Platelets from Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

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SUMMARY The aggregation properties of washed SHRSP platelets were investigated in comparison with normotensive WKY platelets at prehypertensive (4 weeks), early hypertensive (11 weeks) and late hypertensive (17 weeks) ages in the absence of plasma factors. The number of platelets in SHRSP was markedly lower with the development of hypertension than that in WKY. The thrombin- and collagen-induced aggregation was markedly reduced in the platelets from 11 and 17 week old SHRSP compared with that of age-matched WKY, whereas the degree of platelet aggregation in 4 week old SHRSP showed a tendency to be even greater than that in WKY. The changes in blood pressure and platelet aggregability were correlated inversely. ADP did not induce aggregation in the same system used for thrombin and collagen stimulation but in another system it aggregated washed rat platelets. Aggregation responses to ADP and ionophore A23187 were also significantly lower in 14 week old SHRSP platelets than age-matched WKY platelets. Together with other evidence, these results suggest that defective Ca2+ function, rather than the presence of exhausted platelets, is responsible for hypoaggregability in SHRSP platelets.

The paper shows a marked reduction in aggregation responses of washed SHRSP platelets to these agents with the development of hypertension.

Methods

Experimental Animals

SHRSP and WKY were provided by Professor K. Okamoto of Kinki University Medical School and have been maintained by brother-sister breeding in our laboratory. Animals had free access to a laboratory chow (CMF, Oriental Yeast Co., Tokyo) and water, and were killed at prehypertensive (4–5 weeks old), early hypertensive (11 weeks old) and late hypertensive (17 weeks old) ages without a prior fast. Blood was removed from the abdominal aorta under light anesthesia with ether.

Measurement of Blood Pressure

Mean blood pressure was measured by the tail pulse pick-up method in unanesthetized rats after warming the whole body for about 10 min in a chamber maintained at 37°C.

Haematological Examination

The number of red blood cells and platelets, and hematocrit values in a small portion of whole blood were measured by Coulter Counter Model SP (Coulter Electronics Inc., Hialeah, Florida, USA). The number of platelets in a platelet suspension was also counted using the same equipment.

Preparation of Washed Platelets

Washed platelets were prepared principally according to Baenziger and Majerus unless otherwise described. Blood was collected into a siliconized centrifuging tube containing one portion of acid-citrate-dextrose anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose) to five portions of blood, and the mixture was consecutively centrifuged for 15 min at 230 × g (1200 rpm) and for 3 min at 800 × g (2200 rpm) at room temperature. This two step
centrifugation was employed to prevent a loss of platelets with larger sizes. PRP (platelet-rich plasma) separated from the two centrifugations were mixed, and centrifuged for 7 min at 120 × g (800 rpm) to sediment red and white cells. The supernatant was then centrifuged for 15 min at 1700 × g (3200 rpm). The sedimented platelets were resuspended in the same volume of a washing buffer (0.113 M NaCl, 4.3 mM K,HPO4, 4.3 mM Na,HPO4, 24.4 mM NaH2PO4, 5.5 mM glucose, 1 mM EDTA, pH 6.5) as of the whole blood and centrifuged for 15 min, at 1500 × g (3000 rpm) at 4°. The washed platelets were resuspended in a resuspending buffer (0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose, pH 7.4) to make a suspension of about 4 × 10^8 cells/ml. The washed platelet suspensions were kept at 4° until used within 4 h to be protected against the loss of aggregability. The intactness of platelets was ensured throughout all the preparatory stages by measuring the release of lactic acid dehydrogenase activity in the supernatant. For the measurement of ADP-induced aggregation, washed platelets were prepared according to Mustard et al. 19 Briefly, platelets were washed firstly with Ca2+ free Tyrode solution containing bovine albumine (3.5 g/l, Sigma, St. Louis, Mo., USA), heparine (25000 U/l, Sigma Pharmaceuticals, Shimizu, Japan) and apyrase (50 mg/l, from potato, grade 1, Sigma, St. Louis, Mo., USA), and secondly with the above solution deprived of heparine. The washed platelets were resuspended in Ca2+ free Tyrode solution.

Measurement of Platelet Aggregation

The degree of platelet aggregation was measured by the turbidimetric method using 4 channeled NKK Hematrac 1 (Niko Bioscience, Tokyo). To 200 μl of platelet suspension (adjusted to contain about 4 × 10^8 cells/ml) 7 μl of CaCl2 (50 mM) was added to give a final concentration of 1.5 mM Ca2+. Twenty μl of various concentrations of thrombin or collagen were added after 1 min incubation at 37° with constant stirring at 1000 rpm, and changes in turbidity were recorded. Thrombin-induced aggregation were measured 3 min, and collagen- and ionophore A23187-induced aggregation, 5 min respectively after the addition of the aggregating agents. Human plasma thrombin (Midori Cross Co., Osaka, Japan), ADP (from equine muscle, sodium salt, grade 1, Sigma, St. Louis, Mo., USA) and ionophore A23187 (Calbiochem, La Jolla, Ca., USA) were diluted with the resuspending buffer. The solution of collagen (Type 1, insoluble, Sigma, St. Louis, Mo., USA) was prepared according to Inoshita et al., 20 and collagen in the solution was determined by the method of Itzhaki et al. 21 ADP-induced aggregation was measured in the presence of fibrinogen (0.4 mg/ml, human, Sigma, St. Louis, Mo., USA), and a maximum aggregation was read.

Assay of Cholesterol and Protein in Platelets

Platelets were submitted to freeze-thawing and cholesterol was measured using Cholesterol Enzymatic Color Test (Boehringer Mannheim GmbH, Mannheim, Germany). Protein were determined according to Lowry. 22

Results

Blood Pressure

The blood pressure of male SHRSP were not different from that of WKY at 4 weeks, whereas it rose to 210–220 mm Hg at 11 weeks and 17 weeks, and to 248 mm Hg at 25 weeks (figs. 1 and 7). The blood pressure of WKY was always around 130 mm Hg. The ratios of heart to body weight became significantly greater in SHRSP with the development of hypertension.

Haematological Characteristics

The number of platelets at a prehypertensive age was not significantly different between SHRSP and WKY, while the difference became marked with the development of hypertension. The number in SHRSP was reduced to almost one half of WKY at ages of 11, 17 and 26 weeks (fig. 1). Red blood cell counts were slightly greater in 5 and 11 week old SHRSP than in age-matched WKY. No differences in hematocrit values were observed between the two strains throughout all the ages. The protein content (μg/10^8 cells) in SHRSP platelets was almost the same as that in WKY at 5 weeks; while it showed a tendency to becoming greater at 11 weeks, and it became significantly greater by 22% compared with that of WKY at 17 weeks (fig. 2). This result is compatible with the finding that the volume distribution of SHRSP platelets shifts into a larger range. 8 There was no difference in the cholesterol content.

Standard Assay for Washed Platelet Aggregation

The optimal concentrations of aggregating agents and Ca2+ were investigated in washed platelets from WKY. Figure 3a) shows maximum platelet aggregation at varying concentrations of thrombin and collagen in the presence of 1.5 mM Ca2+. Thrombin-induced aggregation reached a maximum at 3 min, and

![Figure 1. Basic characteristics of SHRSP and WKY at prehypertensive, early hypertensive and late hypertensive ages. Error bars indicate standard errors for the number of rats shown in brackets. ** Significance p < 0.01, * Significance p < 0.05.](http://stroke.ahajournals.org/)
the degree of aggregation was dose-dependent in the range of 0.1–0.4 U thrombin/ml, while collagen-induced aggregation reached a maximum at 5 min and was dose-dependent at the concentration of 10–150 μg collagen/ml. Figure 3b) shows the dependency of washed platelet aggregation of extracellular Ca²⁺. An addition of Ca²⁺ appeared to be essential to evoke aggregation. The maximum effect on aggregation occurred at 1–3 mM Ca²⁺. At higher concentrations of Ca²⁺ than 6 mM, aggregation was significantly inhibited. There was no difference in the optimal concentration of Ca²⁺ between SHRSP and WKY platelets. The population of platelets in suspension did not influence platelet aggregability within the range of 2 to 6 × 10⁸ cells/ml under these conditions.

Thrombin- and Collagen-induced Aggregation in SHRSP and WKY at Various Ages

Figure 4 shows typical aggregation curves of platelets from SHRSP and WKY at the ages of 4, 11 and 17 weeks at one fixed concentration of thrombin (0.22 U/ml) and collagen (16.7–18.7 μg/ml), while figure 5 shows the aggregation response of platelets from 11 week old SHRSP and WKY to three different concentrations of the aggregating agents. The degree of aggregation in response to thrombin and collagen in WKY platelets did not change with age (a slight decrease of collagen-induced aggregation at 11 and 17 weeks compared with that at 4 weeks is due to the difference in the concentration of collagen). Aggregation of platelets from 11 and 17 week old SHRSP, however, was markedly reduced in comparison with that of 4 week old SHRSP, and great differences in aggregability were thereby observed between SHRSP and WKY at the ages of 11 and 17 weeks, contrasting with no difference at 4 weeks (fig. 4). The doses of aggregating agents which induced 50% aggregation were thrombin; 0.15 U/ml in WKY and 0.29 U/ml in SHRSP, and collagen; 12 μg/ml in WKY and 42.2 μg/ml in SHRSP, at the age of 11 weeks. Aggregation of SHRSP and WKY at three ages and in three concentrations of aggregators is shown statistically in figure 6. It should be noted that the aggregability of SHRSP is even slightly greater at 4 weeks than that of WKY, but this situation is completely reversed at 11 and 17 weeks. Figure 7 demonstrates a correlated change between aggregation and blood pressure in SHRSP and WKY. The change in platelet functions in SHRSP coincides with the development of hypertension, suggesting that hypoaggregability in SHRSP is secondary to hypertension.

ADP-induced Aggregation in 14 Week Old SHRSP and WKY

Thrombin²³ and collagen²⁴ cause ADP release from platelets, and this ADP is reported to be responsible for platelet aggregation. So decrease in the release reaction to these agents and also decrease in response to released ADP are assumed to be attributable to the
hypoaggregability of SHRSP platelets. Thus, aggregation responses to ADP were examined. Washed rat platelets prepared according to Baenziger and Majerus¹⁸ did not aggregate with ADP (~30 μM) in the same condition as used for thrombin- and collagen-induced aggregation. This will imply that released ADP and fibrinogen from platelets stimulated with thrombin and collagen are not involved in aggregation in this system. On the other hand, washed platelets prepared by the method of Mustard et al.¹⁹ dose-dependently responded to ADP in the presence of fibrinogen. Figure 8a) shows a typical tracing of ADP (2.6 μM)-induced aggregation of platelets from SHRSP and WKY at the age of 14 weeks. In figure 8b) aggregation responses to three doses of ADP (1.3, 1.7, 2.6 μM) were compared between SHRSP and WKY. Aggregation of SHRSP platelets in response to ADP was markedly reduced compared with WKY platelets, as observed in response to thrombin and collagen. The dose of ADP to induce 50% aggregation was 2.2 μM in WKY platelets and 3.3 μM in SHRSP platelets. The result indicates that SHRSP platelets shows hypoaggregability irrespective of proaggregators.

Ionophore A23187-induced Aggregation in 14 Week Old SHRSP and WKY

A rapid increase in intracellular Ca²⁺ concentration following stimulation plays an important regulatory role in excitable cells.²⁵ All three aggregating agents, thrombin, collagen and ADP used in this study, required an addition of Ca²⁺ into the medium to evoke aggregation of washed rat platelet, and notable differences in aggregation response to these proaggregators.

**Figure 4.** Typical aggregation tracings of washed platelets from SHRSP and WKY at the ages of 4, 11 and 17 weeks. Platelets pooled from 2-3 rats were stimulated with the indicated concentrations of thrombin or collagen in the presence of 1.5 mM Ca²⁺.

**Figure 5.** Aggregation responses to three different concentrations of thrombin and collagen from a typical experiment with 11 week old SHRSP and WKY. Ca²⁺ concentration is 1.5 mM.

**Figure 6.** Aggregability of washed platelets from 4, 11 and 17 week old SHRSP and WKY in response to three different concentrations of thrombin and collagen. Washed platelets (4 × 10⁸ cells/ml) were stimulated in the presence of 1.5 mM Ca²⁺. Each column and error bar indicate the mean and standard error of maximum aggregation for the number of platelet preparations shown at the bottom of each column. ** Significance p < 0.01.

**Figure 7.** Changes in blood pressure and platelet aggregability in SHRSP and WKY at various ages. a) Changes in blood pressure, b) and c) changes in aggregability in response to thrombin (b) and collagen (c). Numbers in parentheses indicate the number of platelet preparations.
were observed between SHRSP and WKY with development of hypertension, suggesting that abnormalities either in \( \text{Ca}^{2+} \) transport through plasma membrane or its function may exist in SHRSP platelets. It thus seems interesting to test effects of calcium ionophore A23187 on platelet aggregation of these two strains of the rat. Figure 9a) shows a typical tracing of aggregation of SHRSP and WKY induced by ionophore A23187 (0.3 \( \mu \)M) in the presence of 1.5 mM \( \text{Ca}^{2+} \). Aggregation did not occur in the absence of \( \text{Ca}^{2+} \). A marked difference in aggregation was observed between the two strains in all doses tested (0.2-0.5 \( \mu \)M) (fig. 9b), indicating that abnormalities of SHRSP platelets exist in \( \text{Ca}^{2+} \) functions that is, responsiveness to \( \text{Ca}^{2+} \), rather than in \( \text{Ca}^{2+} \) transport through the plasma membrane.

**Discussion**

With the development of high blood pressure in SHRSP there was a marked decrease in the number of platelets, and aggregation responses of the platelets to thrombin, collagen, ADP and ionophore A23187. As washed platelets were used in this study the influence of plasma factors on platelet functions were excluded. On the basis of the protein content (\( \mu \)g/10^8 cells) the size of SHRSP platelets appeared to become greater with age than that of WKY. This phenomenon might be associated with the shorter survival time of SHRSP platelets with the development of hypertension. These changes in the number and the size of platelets observed by us and others do not seem to result from the exhaustion of platelets due to vascular lesion, because these changes even occurred before the appearance of vascular lesion at early hypertensive ages.

Recently, an acquired platelet dysfunction in man has been observed. It is characterized by defective platelet aggregation, reduced levels of adrenaline nucleotides and serotonin, and an abnormal uptake and storage of amines. This defect is thought to be related to the presence in the circulation of exhausted platelets following their in vivo exposure to inducers of the release reaction, such as damaged endothelium, thrombin and immune complexes. Hypoaggregability of SHRSP platelets might reflect the presence of exhausted platelets. However, this possibility will probably be excluded by the following evidence: 1) that added ADP did not cause aggregation in the system used for thrombin- and collagen-stimulation, 2) that hypoaggregability of SHRSP platelets was observed even at early hypertensive stage, in which no vascular lesion was observed (Yoshimoto H., personal communication), and 3) that our preliminary results showed no differences in ADP and serotonin contents of platelets between SHRSP and WKY at the age of 17 weeks. SHRSP vs WKY mean \( \pm \) S.E. (number of rats): ADP; 0.840 \( \pm \) 0.111 (4) vs 1.04 \( \pm \) 0.093 (5) n mol/10^8 cells, serotonin; 0.687 \( \pm \) 0.048 (9) vs 0.701 \( \pm \) 0.079 (7) n mol/10^8 cells.

Several investigators observed that the cardiovascular system and platelets of SHR responded to \( \text{Ca}^{2+} \) differently from the control. Hamet et al reported hypoaggregability of SHR platelets when they were stimulated with a calcium ionophore A23187. Studies by Shibata and Kurahashi and by Bohr suggested that there was an alteration in \( \text{Ca}^{2+} \)-binding properties of vascular smooth muscle in SHR. In the present study we have shown that aggregation of hypertensive SHRSP platelets were markedly reduced in responses not only to thrombin, collagen and ADP, but also to ionophore A23187, suggesting that abnormal \( \text{Ca}^{2+} \) functions rather than impaired \( \text{Ca}^{2+} \) transport are responsible for the hypoaggregability in SHRSP. We have observed that formation of malondialdehyde which reflects thromboxane A2, a calcium ionophore, formation, was at the same degree in SHRSP platelets as in WKY when stimulated with thrombin. Calcium-calmodulin complex activates myosin light chain kinase as well as many \( \text{Ca}^{2+} \)-dependent enzymes in platelets. The 20,000 dalton light chains of myosin is consequently phosphorylated to form an actin-myosin complex that can hydrolyze ATP and contract. The...
affinity of C-kinase, a calcium dependent protein kinase, to calcium is sharply increased by the presence of diacylglycerols produced through a stimulated phosphatidylinositol-turnover, and the activated enzyme phosphorylates 40,000 dalton protein in platelets preceding the secretion reaction. The secretion of serotonin and adenine nucleotides was also reduced in hypertensive SHRSP platelets (Unpublished data). These evidence support our above assumption. Severe hypoaggregability and reduced number of platelets in addition to mechanical damage of cerebral arterial walls due to hypertension might together contribute to cerebral hemorrhage which occurs in SHRSP in a few months after birth.

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