DYSREGULATION OF Ca\textsuperscript{2+} MOVEMENT IN PLATELETS FROM PATIENTS WITH ACUTE ISCHAEMIC STROKE

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SUMMARY

1. Platelets play a pivotal role during acute ischaemic stroke. An increase in cytosolic Ca\textsuperscript{2+} concentrations ([Ca\textsubscript{2+}]) triggers intracellular signal transduction, leading to platelet aggregation and thrombosis. In the present study, we examined the differences between platelets from acute ischaemic stroke patients and at-risk controls in terms of the increase in platelet [Ca\textsubscript{2+}].

2. Thirty-one patients with acute ischaemic stroke and 27 at-risk controls were enrolled in the present study. Platelet [Ca\textsubscript{2+}], was measured using the fluorescent dye fura-2 after stimulation with 100 \mu mol/L arachidonic acid (AA), 10 \mu mol/L ADP, 1 \mu mol/L platelet-activating factor (PAF) and 0.1 U/mL thrombin.

3. Basal [Ca\textsubscript{2+}], was higher in the stroke group compared with at-risk controls, irrespective of the presence or absence of extracellular Ca\textsuperscript{2+}. In Ca\textsuperscript{2+}-containing medium, both PAF and ADP, but not AA and thrombin, significantly increased platelet [Ca\textsubscript{2+}] in the stroke group compared with the at-risk controls. However, in Ca\textsuperscript{2+}-free medium, only PAF significantly increased platelet [Ca\textsubscript{2+}], in the stroke group compared with the at-risk controls. Basal [Ca\textsubscript{2+}], and PAF-induced platelet [Ca\textsubscript{2+}], increases were still higher in the stroke group at the subacute stage than in the at-risk controls.

4. The results of the present study provide direct evidence that Ca\textsuperscript{2+} signalling in platelets from acute ischaemic stroke patients was altered in response to particular stimuli. The dysregulation of Ca\textsuperscript{2+} movement in platelets may persist up to the subacute stage of ischaemic stroke.

Key words: ADP, calcium, ischaemic stroke, platelet-activating factor, platelets.

INTRODUCTION

Stroke is third most common leading cause of death worldwide and is the major cause of serious long-term disability in adults. Atherothrombosis is the main mechanism of acute ischaemic stroke.

Platelets play a pivotal role in triggering arterial thrombosis and in the progression of atherosclerosis. Increasing evidence demonstrates that the level of platelet activation markers is elevated after acute ischaemic stroke. Thus, platelets are major cellular components involved in atherothrombotic ischaemic stroke.

The molecular mechanisms underlying platelet activation are unclear. It is thought that a transient increase in cytosolic Ca\textsuperscript{2+} concentrations ([Ca\textsubscript{2+}],) plays an important role in mediating transient platelet adhesion, along with sustained calcium signals that are required for irreversible platelet aggregation and thrombosis. The [Ca\textsubscript{2+}], in activated platelets increases due to either Ca\textsuperscript{2+} influx from the extracellular fluid or Ca\textsuperscript{2+} release from intracellular pools. A variety of physiological substances have been shown to increase [Ca\textsubscript{2+}], and affect the physiology of platelets. Thrombin was found to induce granule secretion and platelet aggregation and ADP was found to induce changes in platelet shape and trigger Ca\textsuperscript{2+} mobilization. Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), which is released by activated leucocytes, was found to promote platelet aggregation and arachidonic acid (AA) was shown to elicit changes in platelet shape, serotonin release and platelet aggregation.

Dysregulation of Ca\textsuperscript{2+} movement in platelets has been reported in patients with systemic lupus erythematosus and haemodialysis recipients with occluded vascular access. Previous research has demonstrated that basal platelet [Ca\textsubscript{2+}], is higher in acute stroke patients than in healthy subjects. However, differences in the time-course of platelet Ca\textsuperscript{2+} signalling between stroke patients and at-risk controls have not been investigated completely. In the present study, using the Ca\textsuperscript{2+}-sensitive fluorescent dye fura-2, we tested the hypothesis that, in acute stroke patients, basal platelet [Ca\textsubscript{2+}], increases in the acute stage after stroke and declines thereafter, but remains elevated during the subacute stage, whereas this pattern is not observed in at-risk controls. We also evaluated platelet agonist-induced changes in [Ca\textsubscript{2+}], in both groups.

METHODS

Selection of subjects

We prospectively enrolled consecutive patients with acute ischaemic stroke who presented at the Chang Gung Memorial Hospital–Kaohsiung Medical Center. The study involved two groups of subjects: Group 1 consisted of 31 acute ischaemic stroke patients (acute stroke was defined as a sudden onset of focal cerebral function that persisted for more than 24 h and the diagnosis was established on the basis of medical history, clinical examination and...
results of brain imaging studies; and Group 2 comprised 27 at-risk controls that were age- and gender-matched subjects who had stroke risk factors (hypertension, diabetes, dyslipidaemia or current smoker; see below) without clinical evidence of acute stroke. Risk factors for stroke were defined as follows: hypertension, on antihypertensive treatment or blood pressure > 140/90 mmHg on two measurements; diabetes, on antidiabetic drugs or elevated glycosylated haemoglobin (HbA1c) or blood glucose levels on two readings; and dyslipidaemia, on lipid-lowering medication or total cholesterol > 5.18 mmol/L or triglycerides > 2.03 mmol/L before stroke.21 Demographic and clinical data for both groups were recorded.

Exclusion criteria included primary intracerebral haemorrhage or myocardial infarction that had occurred within the preceding 3 months, existing unstable angina, unstable symptomatic peripheral vascular disease and major surgery or systemic haemorrhage within the preceding 3 months. Moreover, subjects with systemic vasculitis, severe liver disease, end-stage renal disease, underlying neoplasm or known haematological disorders that affected platelet function were excluded from the study. Patients who showed evidence of an infection 1 week before the stroke, those with an elevated body temperature (37.5°C) and/or those who exhibited symptoms of infection after the stroke were also excluded from the study.

**Clinical assessment**

Both stroke patients and the at-risk controls were subjected to complete clinical assessment by senior neurologists. Ancillary examinations were performed, including chest radiography, routine brain computed tomography (CT) or magnetic resonance imaging (MRI)/magnetic resonance angiography (MRA), duplex scanning of the carotid arteries, transthoracic colour-coded sonography (TCCS) and routine cardiac analysis consisting of a 12-lead echocardiogram (ECG).

**Treatment**

The therapeutic regimens for the acute ischaemic stroke patients were based on the guidelines of the American Heart Association (AHA)/American Stroke Association (ASA) for the prevention of stroke in patients with ischaemic stroke or transient ischaemic attack.22 In the present study, we only enrolled acute ischaemic stroke patients who received aspirin (100 mg/day) therapy so we could exclude the effects of various antplatelet therapies. Aspirin inhibits cyclo-oxygenases, leading to the formation of thromboxane A2 and prostaglandins, which subsequently cause platelet aggregation.22 Previous studies revealed that baseline [Ca2+]i and agonist-stimulated increases in [Ca2+]i in platelets may be reduced in patients receiving aspirin therapy.24 Thus, all the at-risk controls were also on aspirin (100 mg/day) for various reasons, including the secondary prevention of stroke, cardiovascular disease or intracranial atherosclerosis, and any contribution of aspirin therapy to platelet [Ca2+], between the two groups could be discounted.

**Blood sampling and assessment of [Ca2+]i**

In the case of stroke patients, blood samples were collected within 48 h of the onset of stroke and in the subacute stage (1 month) after the occurrence of acute ischaemic stroke. Blood samples were obtained once from the at-risk controls, at the time of enrolment. For both the groups, venipuncture of the forearm was performed with the patients in a fasting, non-sedated state between 0900 and 1000 hours to exclude the possible influence of circadian variations. At the time of the first [Ca2+]i measurement, we also determined biochemical data, including the white blood cell (WBC) count, red blood cell (RBC) count, platelet count, activated partial thromboplastin time (APTT), prothrombin time (PT), serum Ca2+ levels, HbA1c levels, total cholesterol and total triglyceride. The study protocol was approved by the Institutional Review Committee on Human Research at the Chang Gung Memorial Hospital–Kaohsiung Medical Center. The protocol for the preparation of platelets and [Ca2+]i measurement was modified according to previous studies.26 Blood samples (8 mL) were drawn from each subject. Cell density was determined by a blood cell counter (K 4500; Sysmex, Hyogo, Japan). Fura-2/acetoxymethyl (final concentration 2 μmol/L; Dozin Chemical Laboratories, Kumamoto, Japan) was added to the platelet-rich plasma (PRP) obtained from 1 : 9 citrated blood and the mixture was incubated at 37°C for 20 min. After centrifugation (600 g, 5 min, 20°C), the supernatant was discarded and the platelet pellet was resuspended in a Ca2+-containing medium (composition (in mmol/L): NaCl 140; KCl 5; MgCl2 1; CaCl2 2; HEPES 10; glucose 5, pH 7.4) at a final platelet count of 2 × 10^8 /μL and maintained at 37°C. The Ca2+ concentration of the solution was adjusted to 1 mmol/L by the addition of CaCl2. The fluorescence of fura-2 was measured at dual excitation wavelengths of 340 and 380 nm and at an emission wavelength of 510 nm using a fluorophotometer (Shimadzu, Kyoto, Japan).

Basal fluorescence (F0) was first measured for a minimum of 30 s, followed by measurement of the maximal change in fluorescence (Ft) after the addition of various stimuli (including 100 μmol/L AA, 10 μmol/L ADP, 1 μmol/L PAF and 0.1 U/mL thrombin). Furthermore, F0 and Ft values were determined after the addition of various stimuli in Ca2+-free medium (the Ca2+ in the aforementioned medium was replaced by 0.3 mmol/L EGTA). Each test was repeated three times. The average net increase in platelet [Ca2+], peak [Ca2+]i, and basal [Ca2+]i was recorded.

In addition, platelets were immersed in 0.1% Triton X-100 and maximum fluorescence was measured at excitation wavelengths of 340 and 380 nm (Fmax-340 and Fmax-380, respectively) after the addition of 5 mmol/L Ca2+. Subsequently, the minimum fluorescence was measured at 340 and 380 nm (Fmin-340 and Fmin-380, respectively) after the addition of 10 mmol/L EGTA (pH > 8.3). Platelet [Ca2+]i, was calculated using the following formula:

\[
\text{Basal [Ca}^{2+}] = k_{d} \times (F_{0} - F_{\text{min-340}})/(F_{\text{max-340}} - F_{0}) \times (F_{\text{max-380}}/F_{\text{max-340}})
\]

Stimulated [Ca2+]i = k_{d} \times (F_{0} - F_{\text{min-340}})/(F_{\text{max-340}} - F_{0}) \times (F_{\text{max-380}}/F_{\text{max-340}})

where k<sub>d</sub> is the dissociation constant for fura-2.

**Statistical analysis**

Biochemical data and platelet [Ca2+]i are expressed as the mean±SEM. Statistical comparisons between the two groups were made using an independent t-test. Paired t-tests were used to evaluate the different time points after acute ischaemic stroke. Fischer’s exact test was used to analyse proportional data. Analysis of covariance (ANCOVA) was used to compare the groups after controlling for potential confounding variables. Levene’s test of equality of error variance was used to ensure equal variance existed in both groups. For comparison of platelet [Ca2+]i, between the groups, ANCOVA was used, with blood pressure and HbA1c as covariates. Differences were considered significant at P < 0.05.

**RESULTS**

General characteristics and laboratory data for both groups are given in Table 1. There were no significant differences between the two groups in terms of age, sex, vascular risk factors (hypertension, diabetes mellitus, dyslipidaemia, cardiac disease and current smoking status), medical treatments and body temperature. Blood pressure (BP) on admission was significantly higher in the acute stroke group than in the at-risk control group. Total serum cholesterol and HbA1c levels showed a similar trend as BP. However, total serum Ca2+ levels were significantly lower in the acute stroke group than in the at-risk control group. The WBC, RBC and platelet counts and serum tri-glyceride levels did not differ significantly between the two groups.

Changes in the platelet [Ca2+]i, after stimulation with 100 μmol/L AA, 10 μmol/L ADP, 1 μmol/L PAF and 0.1 U/mL thrombin are shown in Fig. 1. Basal platelet [Ca2+]i, in the at-risk controls and in stroke patients at the acute and subacute stages are shown in Fig. 2. The data reveal that basal platelet [Ca2+]i, was higher in the acute
stroke group compared with the at-risk control group, irrespective of the presence or absence of Ca\(^{2+}\) in the suspension medium. In order to exclude the possible effects of BP and serum glucose on platelet [Ca\(^{2+}\)], we tested the hypothesis that platelet [Ca\(^{2+}\)], was equal between BP and blood glucose by using ANCOVA. The variation in platelet [Ca\(^{2+}\)], was determined to be equal between groups through Levene’s test of equality of error variance \(P = 0.35\) and 0.84 in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium, respectively. Univariate analysis of covariance demonstrated that platelet [Ca\(^{2+}\)], levels between the two groups were statistically different \((P < 0.01\) in Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free medium). Basal platelet [Ca\(^{2+}\)], was lower in the subacute stage than in the acute stage in stroke patients. However, basal platelet [Ca\(^{2+}\)], was still significantly higher in stroke patients at the subacute stage than in the at-risk controls, irrespective of the presence or absence of Ca\(^{2+}\) in the suspension medium.

The net increase in platelet [Ca\(^{2+}\)], after stimulation by various agonists during the acute and subacute stages is shown in Fig. 3. These values were significantly higher in the acute stroke group than in the at-risk control group after stimulation with PAF and ADP, but not after stimulation with AA and thrombin, in Ca\(^{2+}\)-containing medium (Fig. 3a). After stimulation with AA, ADP and PAF, the net increase in platelet [Ca\(^{2+}\)], was similar in platelets from the acute and subacute stages. Furthermore, the PAF-induced increase in platelet [Ca\(^{2+}\)], was significantly higher in the stroke group at the subacute stage than in the at-risk control group. Similar experiments were performed in Ca\(^{2+}\)-free medium in order to determine whether the stimuli induced an increase in platelet [Ca\(^{2+}\)], via Ca\(^{2+}\) influx from the extracellular space or as a result of its release from Ca\(^{2+}\) pools (Fig. 3b). In stroke patients, the net increase in platelet [Ca\(^{2+}\)], after PAF stimulation was significantly higher in both the acute and subacute stages compared with at-risk controls. However, there was no difference in the net increase in platelet [Ca\(^{2+}\)], between the acute stroke patients and at-risk controls after stimulation with AA, ADP and thrombin.

## DISCUSSION

The present study demonstrates that basal platelet [Ca\(^{2+}\)], is higher in acute ischaemic stroke patients than in at-risk controls. Basal platelet [Ca\(^{2+}\)], remained relatively high during the subacute stage of ischaemic stroke. Previous research has shown that [Ca\(^{2+}\)], plays a pivotal role in signal transduction in platelets. The increases in platelet [Ca\(^{2+}\)], are involved in the major responses of platelets to stimulation, including changes in shape, aggregation and secretion. These results indicate that the increase in basal platelet [Ca\(^{2+}\)], in stroke patients represents a lower threshold for platelet activation during the acute and subacute stages.

In order to estimate the mechanism responsible for the elevation of platelet [Ca\(^{2+}\)], four general endogenous hormones (AA, ADP, PAF and thrombin) were used as platelet stimulators. Ca\(^{2+}\) influx was

### Table 1  Baseline characteristics and laboratory data of stroke patients and at-risk controls

<table>
<thead>
<tr>
<th></th>
<th>At-risk controls ((n = 27))</th>
<th>Stroke patients ((n = 31))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63.0 ± 1.9</td>
<td>66.6 ± 1.8</td>
</tr>
<tr>
<td>Percentage of men ((n))</td>
<td>64.3 (18)</td>
<td>64.5 (20)</td>
</tr>
<tr>
<td>Percentage with hypertension ((n))</td>
<td>88.9 (24)</td>
<td>77.0 (24)</td>
</tr>
<tr>
<td>Percentage with diabetes mellitus ((n))</td>
<td>44.4 (12)</td>
<td>41.9 (13)</td>
</tr>
<tr>
<td>Percentage with dyslipidaemia percentage ((n))</td>
<td>40.7 (11)</td>
<td>25.8 (8)</td>
</tr>
<tr>
<td>Percentage with cardiac disease ((n))</td>
<td>7.4 (2)</td>
<td>9.7 (3)</td>
</tr>
<tr>
<td>Percentage current smokers ((n))</td>
<td>25.9 (7)</td>
<td>25.8 (8)</td>
</tr>
<tr>
<td>Percentage with previous history of stroke ((n))</td>
<td>37.0 (10)</td>
<td>29.0 (9)</td>
</tr>
<tr>
<td>Percentage on antiplatelet therapy ((n))</td>
<td>100 (27)</td>
<td>100 (31)</td>
</tr>
<tr>
<td>Percentage on antihypertensive therapy ((n))</td>
<td>81.4 (21)</td>
<td>77.0 (24)</td>
</tr>
<tr>
<td>Percentage on antidiabetic therapy ((n))</td>
<td>44.4 (12)</td>
<td>38.7 (12)</td>
</tr>
<tr>
<td>Percentage on lipid-lowering therapy ((n))</td>
<td>33.3 (9)</td>
<td>25.8 (8)</td>
</tr>
<tr>
<td>SBP on admission (mmHg)</td>
<td>137.5 ± 3.1</td>
<td>149.0 ± 3.9*</td>
</tr>
<tr>
<td>DBP on admission (mmHg)</td>
<td>74.9 ± 2.4</td>
<td>88.6 ± 2.1**</td>
</tr>
<tr>
<td>SBP at 1 month (mmHg)</td>
<td>136.6 ± 2.2</td>
<td>140.1 ± 2.3</td>
</tr>
<tr>
<td>DBP at 1 month (mmHg)</td>
<td>74.1 ± 1.7</td>
<td>77.9 ± 1.3</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>36.5 ± 0.5</td>
<td>36.7 ± 0.5</td>
</tr>
<tr>
<td>Laboratory data</td>
<td></td>
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<tr>
<td>White blood cell count (x10^3/(\mu)L)</td>
<td>6.4 ± 0.3</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>Red blood cell count (x10^3/(\mu)L)</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>Platelet count (x10^3/(\mu)L)</td>
<td>20.6 ± 1.0</td>
<td>19.8 ± 1.3</td>
</tr>
<tr>
<td>PT (s)</td>
<td>9.6 ± 0.1</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>28.7 ± 0.4</td>
<td>27.8 ± 0.5</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>173.6 ± 7.3</td>
<td>203.9 ± 10.6*</td>
</tr>
<tr>
<td>Total triglyceride (mg/dL)</td>
<td>147.2 ± 24.0</td>
<td>168.1 ± 32.5</td>
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<tr>
<td>HbA1c (%)</td>
<td>6.7 ± 0.3</td>
<td>8.0 ± 0.5*</td>
</tr>
<tr>
<td>Serum Ca(^{2+}) (mg/dL)</td>
<td>9.0 ± 0.1</td>
<td>8.6 ± 0.1**</td>
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</table>

Where appropriate, data are given as the mean±SEM. *\(P < 0.05\), **\(P < 0.01\) compared with at-risk controls.

SBP, systolic BP; DBP, diastolic BP; PT, prothrombin time; APTT, activated partial thromboplastin time; HbA1c, glycosylated haemoglobin.
influx of Ca^{2+} from the external medium is the source of this enhancement. Conversely, PAF-induced Ca^{2+} signals were enhanced under both conditions, indicating that Ca^{2+} is released primarily from intracellular stores, although a minor increase in Ca^{2+} influx is possible. Different stimulators activate human platelets via different receptors and signal pathways. For example, ADP activates human platelets through several P2 (P2Y1, P2Y12 and P2X1) receptors, resulting in calcium mobilization, thromboxane A2 production and changes in platelet shape.27,28 Platelet-activating factor functions by binding to a unique G-protein-coupled receptor, activating multiple intracellular signalling pathways and stimulating Ca^{2+} mobilization via a receptor-operated channel.16,29 Thus, the substantial PAF- and ADP-associated increases in [Ca^{2+}]i in acute stroke patients suggest that the properties of platelet receptors change after ischaemic stroke. Further studies are required to identify the mechanisms that underlie the altered platelet [Ca^{2+}]i after ischaemic stroke.

An interesting finding of the present study is that serum [Ca^{2+}]i was significantly lower in the acute ischaemic stroke group compared with the at-risk control group, whereas basal [Ca^{2+}]i was higher in the acute stroke group than in the at-risk control group. Previous studies demonstrated that a higher serum Ca^{2+} level at admission is associated with lesser stroke severity, smaller cerebral infarct volume and better functional outcome.30,31 These results suggest that the
In conclusion, in patients with acute ischaemic stroke, PAF-induced increases in platelet [Ca\(^{2+}\)], occurred as a result of enhanced Ca\(^{2+}\) release from intracellular stores, whereas the ADP-induced increase in platelet [Ca\(^{2+}\)], was a result of enhanced Ca\(^{2+}\) influx. The dysregulation of Ca\(^{2+}\) movement in platelets may persist up to the subacute stage after ischaemic stroke. The present study also provides evidence that Ca\(^{2+}\) signalling in platelets from acute stroke patients was altered in response to particular stimuli.

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