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Platelet adhesion and intracellular calcium levels in antigen-challenged rats

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ABSTRACT

There is considerable evidence that platelet activation occurs in allergic airways diseases. In this study we aimed to investigate platelet adhesion to immobilized fibrinogen and intracellular calcium levels in a rat model of allergic inflammation. Male Wistar rats were challenged with ovalbumin (OVA). At 30 min to 24 h after OVA-challenge, assays of platelet adhesion to immobilized fibrinogen and intracellular calcium levels using fura 2-AM loaded platelets were performed. The serum levels of IgE were approximately 5-fold greater in OVA-sensitized rats. A marked eosinophil influx in bronchoalveolar lavage (BAL) fluid of OVA-challenged rats at 24 h after OVA-challenge was also seen. OVA-challenge resulted in a marked thrombocytopenia, as observed within 12 h after OVA-challenge. The agonists ADP (0.5–50 μM) and thrombin (30–100 mU/ml) concentration-dependently increased platelet adhesion to immobilized fibrinogen. At an early time after OVA-challenge (30 min), platelets exhibited greater platelet adhesion compared with the non-sensitized group, whereas at a late time (24 h) they exhibited lower platelet adhesion to both agonists. Moreover, at 30 min after OVA-challenge, intracellular calcium levels to ADP (20 μM) and thrombin (100 mU/ml)-activated platelets were greater compared with non-challenged rats. As opposed, at 24 h after OVA challenge, a lower intracellular calcium level to ADP- and thrombin-activated platelets was observed. In conclusion, OVA-challenge in rats promotes a biphasic response in platelet adhesion consisting of an increased adhesion and intracellular calcium levels at an early phase (30 min), which progress to a reduction in adhesion and intracellular calcium levels at a late time (24 h) after antigen challenge.

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1. Introduction

Platelets are anucleate blood elements derived from megakaryocytes in the bone marrow. Platelet adhesion to extracellular matrix proteins such as collagen, fibronectin, immobilized fibrinogen and von Willbrand factor is considered one of the initial steps of platelet activation for haemostasis maintenance [1]. Platelets have also been reported to affect induction and maintenance of inflammatory and immunological reactions [2]. Platelets possess many of the features of classical inflammatory cells such as polymorphonuclear leucocytes, and under activation release secretory products and express immune receptors on their membrane, with resultant induction of biological activities including cell adhesion, chemotaxis, cell survival and proliferation [3].

Changes in platelet behavior and function have also been documented in a wide variety of inflammatory disorders including allergic diseases in animals and humans [2,4]. In humans, a previous study demonstrated that nocturnal asthma is accompanied by platelet activation and increases in bronchial reactivity [5]. Increased plasma levels of β-thromboglobulin (β-TG) and platelet factor-4 (PF4) were also found in symptomatic asthmatic patients in comparison with healthy controls [6]. A kinetic of platelet activation following allergen provocation of allergic asthmatics was carried out to determine the dynamics of platelet activation relative to changes in lung function and changes in airway inflammation [7]. These authors observed a thrombocytopenia following allergen challenge in atopic asthmatics that persists for 24 h, a time when bronchoconstriction has resolved, but when inflammatory changes persist. In murine models of allergic inflammation, the platelet P-selectin adhesion molecule was shown to mediate the platelet–leukocyte interaction, which is required for trafficking of eosinophils and lymphocytes into the lung tissue [8]. In addition, allergen-induced bronchoconstriction in mice is accompanied by migration of platelets to lung tissue [9]. Platelets from ovalbumin-sensitized mice were shown to induce leukocyte recruitment into skin by forming platelet–leukocyte complexes via P-selectin in circulating blood and secreting chemokines that attract leukocytes to skin [4]. Although platelets are reported to act as innate inflammatory cells in immune responses, and become activated upon allergen exposure [10], studies focusing on the

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mechanisms by which platelets become activated in allergic airways diseases are still scarce, and few studies exist evaluating platelet adhesion in allergic animals [11,12]. Therefore, this study aimed to explore the relationship between platelet activation and allergic inflammation, looking at both the *ex-vivo* platelet adhesion to immobilized fibrinogen and intracellular calcium levels in a rat model of allergic inflammation.

2. Materials and methods

2.1. Animal experimentation guidelines

The experimental protocols were approved by the Ethical Principles in Animal Research adopted by The Brazilian College for Animal Experimentation (COBEA). Male Wistar rats were housed in temperature-controlled rooms and received water and food *ad libitum* until used.

2.2. Sensitization and ovalbumin (OVA) challenge procedures

Adult rats (180–200 g) were sensitized with OVA chicken egg (grade III). Sensitization was performed by subcutaneous injection of OVA (0.15 ml) solution containing 200 μg of OVA and 8 mg of Al $(OH)_3$ prepared in saline [13]. Non-sensitized rats received only Al (OH_3). Fourteen days later, sensitized and non-sensitized animals rats were anaesthetized with isoflurane and intranasally exposed to a single challenge of 200 μL of OVA (5 mg/ml) solution in sterile phosphate-buffered saline (PBS). At 30 min to 24 h after OVA instillation in OVA-sensitized and non-sensitized rats, the following assays have been carried out: (a) counts of total and differential leukocytes in bronchoalveolar lavage (BAL) fluid, (b) counts of platelets in peripheral blood, (c) adhesion to immobilized fibrinogen and (d) measurement of intracellular calcium levels in isolated platelets (see details below). A total of 5–10 rats were used for each time-point in each assay.

2.3. Leukocyte counts in BAL fluid

Animals were anaesthetized with isoflurane, and trachea was exposed and cannulated with a polyethylene tube (1-mm diameter) connected to a syringe. The lungs were washed by flushing with PBS solution containing heparin (20 UI/ml). The PBS buffer was instilled through the tracheal cannula as one 10-ml aliquot followed by three 5-ml aliquots. Next, the total fluid recovered after each aliquot instillation was centrifuged (1000*g* for 10 min at 20 °C). The volumes of fluid recovered from non-sensitized rats did not significantly differ from sensitized animals. Cell pellet of each aliquot was pooled in 2 ml of PBS solution. Total cell counts were done on Neubauer chamber while differential counts were carried out on a minimum of 200 cells using cytospin preparation stained with Diff Quick. The cells were classified as neutrophils, eosinophils, and mononuclear cells based on normal morphological criteria.

2.4. Peripheral blood platelet count

Animals were anaesthetized with isoflurane. Aliquots (10 μL) of peripheral blood were collected by tail bleed. The blood was added to 380 μL of ammonium oxalate (1%) plus 10 μL of ACD-C for 10 min and then counted in Neubauer chamber. The values are expressed as number of platelets/mm^3 blood. Repeated blood sampling was made from the tail vein animal 24 h before (zero time) and at 30 min, 1 h, 2 h, 8 h, 12 h and 24 h post-OVA instillation in non-sensitized ($n = 6$) and sensitized rats ($n = 6$). Animals undergoing tail bleeding were not used for other experimental procedures.

2.5. Isolation of blood platelets

Animals were anaesthetized with isoflurane. Rat arterial blood was collected by using a butterfly needle (19G gauge) in the presence of the anticoagulant acid citrate dextrose (ACD-C; citric acid 3%, trisodium citrate 4%, glucose 2%; 1:9 v/v) in a polyethylene tube (15 ml). Blood was centrifuged at 600*g*, 20 °C for 12 min. Platelet-rich plasma (PRP) was washed twice in a wash buffer solution (NaCl 140 mM, KCl 5 mM, sodium citrate 12 mM, glucose 10 mM and saccharose 12 mM; pH = 6; 5:7 v/v), and centrifuged at 800*g*, 20 °C for 13 min. Platelet pellet was gently resuspended (1.2×10^8 platelets/ml) in a free-Ca^{2+} Krebs–Ringer solution. Next, $CaCl_2$ (1 mM) was added to the platelet suspension for using in the adhesion assays, as detailed below. Different group of animals was used to obtain washed platelets for the assays at 30 min, 2 h, 8 h and 24 h time-points after OVA instillation in non-sensitized and sensitized animals.

2.6. Platelet adhesion to immobilized fibrinogen

Platelet adhesion assays were carried out in 96-well microtiter plates coated with fibrinogen [14,15]. Briefly, the 96-well microtiter plates were coated (overnight at 4 °C) by adding 50 μl/well of a fibrinogen solution (50 μg/ml). Before use, the wells were washed twice with Krebs solution. After washing, the non-specific adhesion was blocked by incubation of wells with 1% BSA for 1 h at 37 °C. At the end of incubation plates were washed again. Platelets (50 μl/well; 1.2×10^8 platelets/ml) were stimulated with either thrombin (10–200 mU/ml) or ADP (0.5–50 μM), and allowed to adhere to the wells for 30 min at 37 °C. Thereafter, plates were carefully washed twice with 200 μl/well of Krebs solution to remove unattached platelets. Adherent platelets were quantified by measuring acid phosphatase activity. The platelets were incubated with acid phosphatase substrate solution (0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100) for 1 h at room temperature, after which 100 μL of 2 N NaOH were added to each well. The *p*-nitrophenol produced by the reaction was measured with a microplate reader at 405 nm. The percentage of adherent cells was calculated on the basis of a standard curve obtained with known number of platelets ($0–6 \times 10^6$ platelets/well). All the experiments were performed in triplicates.

2.7. Fura 2-AM loading platelets and Ca^{2+} measurements

Isolated platelets (3×10^8 platelets/ml) were suspended in a free-Ca^{2+} Krebs–Ringer solution in the presence of pluronic F-68 (0.5 mg/l) [16]. Platelets were loaded with fura 2-AM (2 μM) for 45 min at room temperature protected from light. Thereafter, platelet suspension was centrifuged at 700*g* for 10 min added of the prostacyclin analogue iloprost (0.5 μM). The pellet of platelet was resuspended (2×10^8 platelets/ml) in Krebs solution and stored in the dark until Ca^{2+} measurements. Aliquots of platelets (1 ml) were dispensed into cuvettes (Hitachi F-2000, Japan) equipped with a stirring device. To obtain total intracellular calcium levels, the external Ca^{2+} concentration was adjusted to 1 mM with $CaCl_2$, following equilibration for at least 30 s. Next, either thrombin (100 mU/ml) or ADP (20 μM) was added to induce platelet activation. To verify the intracellular Ca^{2+} levels from internal storages sites alone, Ca^{2+}-free Krebs solution in the presence of 2 mM EGTA was used. The fura 2-AM fluorescence was monitored continuously with monochromator settings of 339 nm (excitation) and 500 mM (emission). The external influx of Ca^{2+} was calculated by subtracting the total Ca^{2+} levels from the internal storage mobilization. The intracellular Ca^{2+} levels were calculated by use of a general formula as previously described [17]. Briefly, the $[Ca^{2+}]_i$ in nM was

calculated according to the formula: $[Ca^{2+}]_i = k_d \times (F - F_{min})/(F_{max} - F)$, where k_d (224) is the fura dissociation constant, according to a previous study. The F_{max} value was obtained with triton X-100 (0.1%) in the presence of saturating calcium ($CaCl_2$ 1 mM), whereas F_{min} value was obtained with the calcium chelator EGTA (10 mM) plus Tris (20 mM; pH = 8).

2.8. Serum IgE level measurement

The serum IgE levels in non-sensitized and OVA-sensitized rats were measured using an enzyme-linked immunosorbent assay commercial kit for rat IgE, following the manufacturer instructions (Biovendor Research and Diagnostic Products, Shibayagi Co., Ltd, Japan).

2.9. Materials

Adenosine diphosphate (ADP), bovine serum albumin (BSA), fibrinogen (fraction I from human plasma), fura 2-AM, chicken egg ovalbumin (OVA grade III), phosphatase substrate (*p*-nitrophenyl phosphate), pluronic F-68, and thrombin (from human plasma) were purchased from Sigma Chem. Co. (St. Louis, MO).

2.10. Statistical analysis

Data are presented as the means ± SEM of *n* experiments. The program Instat (GraphPad software) and the SAS System for Windows (version 8.02) were used for statistical analysis. Two-way repeated measures ANOVA followed by Tukey (or Bonferroni) test was used to analyse platelet counts in circulating blood, adhesion, calcium mobilization and cells in BAL fluid. Non-paired Student's *t* test was used to analyse serum IgE levels. A value of $P < 0.05$ was accepted as significant.

3. Results

3.1. Serum IgE levels and leukocyte counts in BAL fluid

The efficiency of OVA sensitization and challenge procedures were assessed by measuring serum IgE levels and leukocyte number in BAL fluid. Serum IgE levels in sensitized rats (1281 ± 113.5 ng/ml; $n = 10$; $p < 0.001$) were significantly greater than non-sensitized animals (264 ± 45.7 ng/ml; $n = 4$).

Examination of BAL fluid at 30 min post-OVA challenge showed no significant changes in the number of neutrophils, eosinophils and mononuclear cells (Table 1). At 24 h post-OVA challenge, a marked accumulation of eosinophils, neutrophils and mononuclear cell number in BAL fluid were seen in comparison with non-sensitized rats (Table 1).

Table 1
Leukocyte counts in bronchoalveolar lavage (BAL) fluid after ovalbumin (OVA) challenge. Total and differential leukocyte counts ($\times 10^6$ cell/ml) were determined in BAL fluid at 30 min and 24 h post-OVA challenge in comparison with non-sensitized (NS) rats. Total and differential leukocyte counts ($\times 10^6$ cell/ml) were also determined in BAL fluid of naïve rats. Values are expressed as means ± S.E.M ($n = 5$ each group). Data analyzed by means of ANOVA followed by the Bonferroni multiple comparison test ($^*p < 0.05$ compared with respective NS group).

Groups		Total cells	MN	Eosinophils	Neutrophils
Naïve rats		0.58 ± 0.08	0.58 ± 0.08	ND	ND
NS	30 min	0.53 ± 0.07	0.53 ± 0.07	ND	ND
OVA	30 min	0.72 ± 0.12	0.70 ± 0.12	ND	0.02 ± 0.01
NS	24 h	0.45 ± 0.08	0.42 ± 0.08	ND	0.03 ± 0.01
OVA	24 h	1.43 ± 0.07*	0.80 ± 0.06*	0.20 ± 0.02*	0.43 ± 0.03*

MN, mononuclear cells. ND, not detected.

3.2. Time-course of platelet counts in peripheral blood

The OVA challenge resulted in a sustained decrease of platelet counts in peripheral blood, starting at 60 min and achieving the maximum decrease at 8 h after OVA challenge. At 24 h post-challenge, the number of circulating platelets nearly normalized, compared with non-sensitized rats (Fig. 1).

3.3. Spontaneous and activated platelet adhesion to immobilized fibrinogen

Platelets obtained both prior to OVA challenge and at 30 min, and 2, 8 and 24 h after OVA challenge were isolated, and adhesion assays to immobilized fibrinogen were carried out.

The spontaneous platelet adhesion (non-activated platelets) remained unchanged in all experimental groups (Table 2). However, addition of ADP (0.5–50 μM) to activate platelets concentration-dependently increased platelet adhesion to immobilized fibrinogen, as assessed in both non-sensitized and OVA-challenged groups. Platelets obtained at 30 min post-OVA challenge exhibited a significantly greater ADP-induced platelet adhesion, compared with those obtained from non-sensitized rats (Fig. 2). At intermediate time-periods after OVA challenge (2 h and 8 h post-OVA challenge), ADP-induced platelet adhesion did not significantly differ between OVA-sensitized and non-sensitized rats (Fig. 2). Rather, at 24 h post-OVA challenge, platelets exhibited a significantly lower platelet adhesion (Fig. 2).

Similarly to ADP, thrombin (30–100 mU/ml) concentration-dependently increased platelet adhesion to immobilized fibrinogen in both non-sensitized and OVA-challenged groups. Platelets obtained at 30 min post-OVA challenge exhibited a significantly greater platelet adhesion to thrombin (Fig. 3). At 2 and 8 h post-OVA challenge, thrombin-induced platelet adhesion did not significantly differ between OVA-sensitized and non-sensitized rats (Fig. 3), whereas at 24 h post-OVA challenge, platelets exhibited a significantly lower platelet adhesion at the dose of 100 mU/ml (Fig. 3).

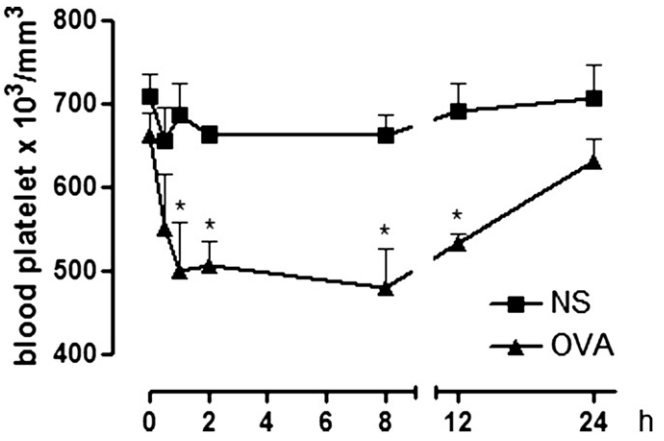


Fig. 1. Time-course of platelet counts in peripheral blood of ovalbumin (OVA)-challenged rats. Aliquots (10 μL) of peripheral blood were collected by tail bleed, before and after OVA-challenge in comparison with non-sensitized (NS) rats. Values are expressed as number of platelets/mm³ blood. Data are expressed as means ± S.E.M ($n = 6$ each group), and analyzed by two-way ANOVA followed by Tukey test. $^*p < 0.05$ compared with time 0 in the same group.

Table 2
Spontaneous platelet adhesion to immobilized fibrinogen prior to ovalbumin (OVA) challenge and after OVA challenge (30 min to 24 h). Non-stimulated platelets (6×10^6 platelets/well) were allowed to adhere to fibrinogen-coated plates in the presence of $CaCl_2$ (1 mM). All the experiments were performed in triplicates. Data are expressed as means ± S.E.M. ($n = 4–8$ each group).

Groups	Adhesion (%)				
	Prior to OVA challenge	Time after OVA challenge			
		30 min	2 h	8 h	24 h
NS	7.5 ± 1.0	8.8 ± 0.8	9.9 ± 0.5	8.7 ± 0.8	7.4 ± 0.8
OVA	6.7 ± 0.5	9.8 ± 1.1	9.6 ± 0.6	8.9 ± 0.8	8.4 ± 1.0

3.4. Intracellular calcium levels in platelets at 30 min after OVA challenge

Fig. 4 shows the intracellular calcium levels in platelets at 30 min after OVA instillation in non-sensitized and sensitized rats. In the presence of 1 mM $CaCl_2$ (Fig. 4A), ADP (20 μM) and thrombin (100 mU/ml) significantly increased the total intracellular calcium levels in platelets obtained from both non-sensitized and sensitized rats compared with non-stimulated platelets. In ADP (but not thrombin)-activated platelets, the total intracellular calcium levels in platelets from OVA-challenged rats were significantly greater compared with those obtained from non-challenged rats (Fig. 4A).

To verify the intracellular Ca^{2+} levels from internal storage sites alone, a Ca^{2+}-free Krebs solution in the presence of EGTA (2 mM) was used. The intracellular calcium levels in non-stimulated platelets were reduced by approximately 70% ($P < 0.05$) in both non-sensitized and sensitized groups, as expected (Fig. 4B). In such conditions, the intracellular calcium levels were greater in ADP- and thrombin-stimulated platelets from OVA-challenged group in comparison with non-sensitized rats instilled with OVA (Fig. 4B).

3.5. Intracellular calcium levels in platelets at 24 h after OVA challenge

Fig. 5 shows the intracellular calcium levels in platelets at 24 h after OVA instillation in non-sensitized and sensitized rats. In the presence of 1 mM $CaCl_2$ (Fig. 5A), ADP (20 μM) and thrombin (100 mU/ml) significantly increased the total intracellular calcium levels in platelets obtained from both non-sensitized and sensitized rats compared with non-stimulated platelets. No significant changes between both of these groups were observed.

In the absence of Ca^{2+} (Ca^{2+}-free Krebs solution plus addition of EGTA), the intracellular calcium levels were markedly reduced ($P < 0.05$) in both non-sensitized and sensitized groups (Fig. 5B) when compared with conditions in the presence of Ca^{2+} (Fig. 5A). In these conditions (absence of Ca^{2+}), significantly lower intracellular calcium levels in both non-stimulated and stimulated platelets (ADP and thrombin) were observed in comparison with the non-sensitized animals instilled with OVA (Fig. 5B).

4. Discussion

The present study shows that OVA challenge in rats promotes a biphasic response in platelet adhesion to immobilized fibrinogen consisting of an increased adhesion at an early phase (30 min) followed by a reduction in adhesion at an late phase after antigen challenge (24 h). At the early phase, there is an enhancement in the intracellular calcium levels in platelets, which progress to a significant drop at the late phase.

A fundamental feature of asthma associated with allergic sensitization is the ability of the airways to recognize allergens and to generate the so-called Th2 cytokines, which is related to IgE production, eosinopoiesis, and bronchial inflammation [18]. According to the experimental model of asthma here employed, sensitization and

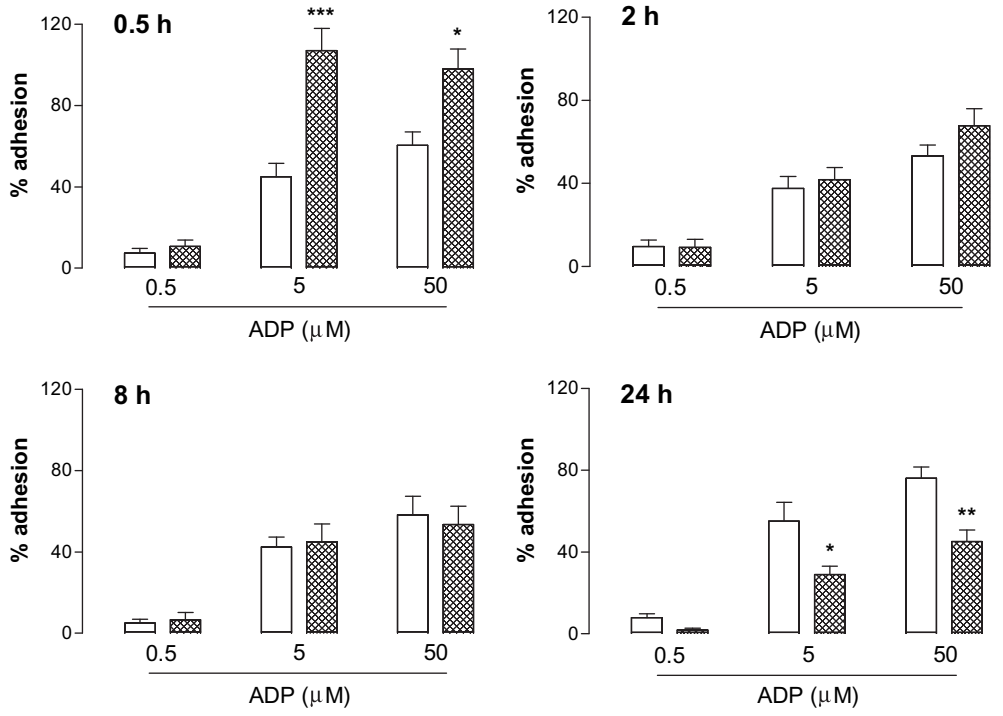


Fig. 2. ADP-induced platelet adhesion to immobilized fibrinogen after ovalbumin (OVA)-challenge. Isolated platelets (6×10^6 platelets/well) from either non-sensitized (open columns) or OVA-challenged rats (cross-hatched columns) were activated with ADP (0.5–50 μM) and allowed to adhere in fibrinogen-coated plates. All the experiments were performed in triplicates. Values are expressed as means ± S.E.M. ($n = 6–8$ each group). Data were analyzed by two-way ANOVA followed by Bonferroni test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with NS group).

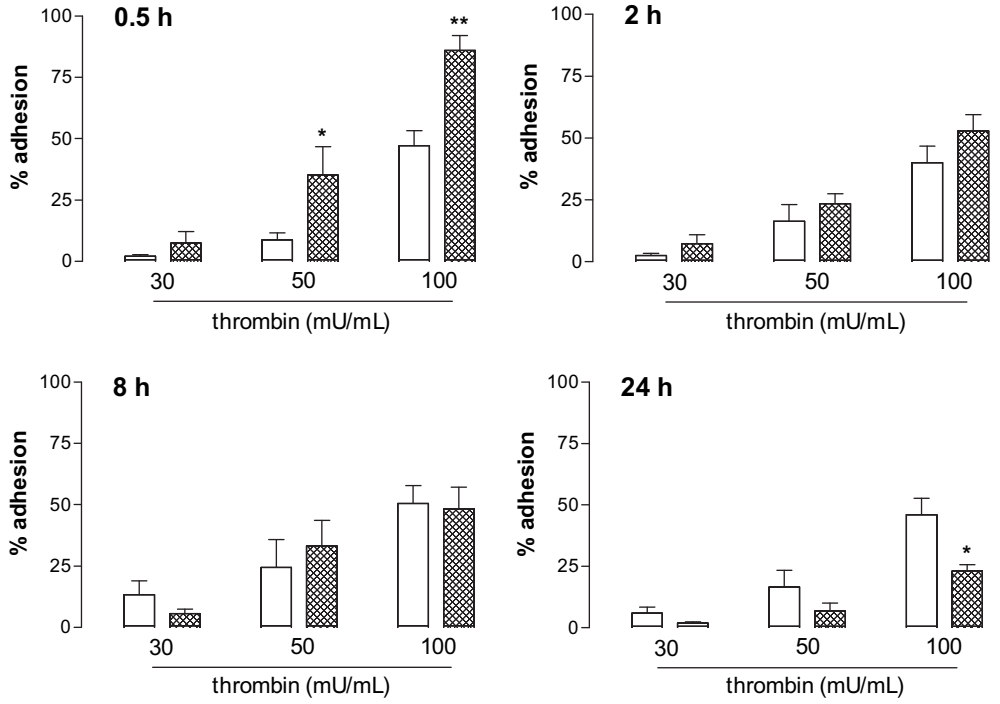


Fig. 3. Thrombin-induced platelet adhesion to immobilized fibrinogen after ovalbumin (OVA)-challenge. Isolated platelets (6×10^6 platelets/well) from either non-sensitized (NS; open columns) or OVA-challenged rats (cross-hatched columns) were activated with thrombin (30–100 mU/ml) and allowed to adhere in fibrinogen-coated plates. All the experiments were performed in triplicates. Values are expressed as means ± S.E.M ($n = 6$–8 each group). Data were analyzed by two-way ANOVA followed by Bonferroni test ($^*p < 0.05$, $^{**}p < 0.01$ compared with NS group).

challenge with OVA in rats typically results in an allergic response characterized by eosinophilia and IgE production. This model in rats is reported to reproduce some of the aspects seen in asthmatic individuals, including the immediate and late asthmatic responses, IgE production, and pulmonary eosinophil inflammation [19,20]. In our study, the serum IgE levels were approximately 5-fold greater in OVA-sensitized rats, as expected. Moreover, the leukocyte profile in BAL fluid showed a late eosinophil influx, as observed at 24 h post-OVA. This reinforces that the rat model of allergy used is suitable to further understand the relationship between allergic inflammation and platelet function. Interestingly, at 24 h after OVA challenge we also observed a large infiltration of neutrophils. Accordingly, current attention has focused on patterns of eosinophil and neutrophil infiltration, where the former are linked with Th2 lymphocyte responses and the latter possibly with dysfunctional innate immune responses. Moreover, non-eosinophilic pattern of response has been associated with more severe persistent asthma, non-response to corticosteroid therapy and certain specific trigger factors such as tobacco smoking and occupational exposures [21].

Clinical evidences have shown that the airway obstruction seen after allergen inhalation in asthmatic individuals is accompanied by thrombocytopenia [7,22]. Accordingly, our results showed a marked reduction in the counts of circulating platelets, as detected as early as 60 min after OVA-challenge, achieving the maximum decrease at 8 h after OVA challenge, and normalizing at 24 h post-challenge. It has been suggested that thrombocytopenia during an asthma attack is the result of migration of platelets from circulation to the airways [9,23]. Moreover, platelets are reported to accumulate in the lung and liver immediately after intravenous injection of OVA in previously sensitized mice that is accompanied by platelet degranulation

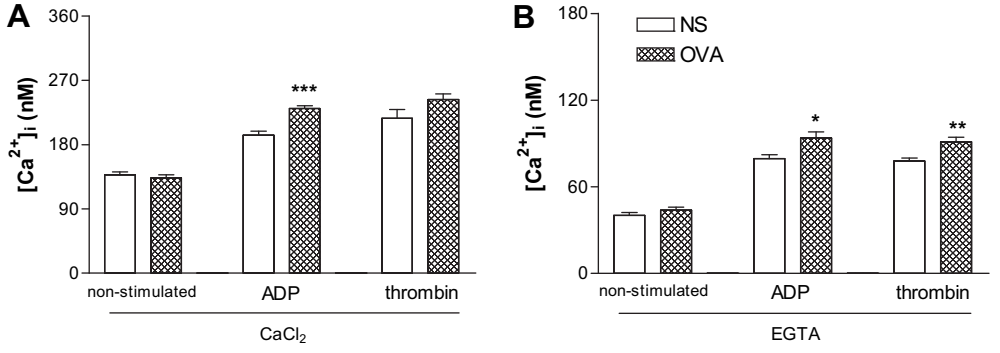


Fig. 4. Enhancement in intracellular calcium levels in ovalbumin (OVA)-challenged rat platelets 30 min after challenge. Changes in total (panel A) and internal Ca^{2+} levels (panel B) in response to either ADP (20 μM) or thrombin (100 mU/ml) in platelets from non-sensitized (NS) and OVA-challenged rats. Internal Ca^{2+} levels were determined in Ca^{2+}-free medium and presence of EGTA (2 mM). Note that Y-axis in panel A varies from zero to 360 nM, whereas in panel B it varies from zero to 180 nM. Values are expressed as mean ± S.E.M. ($n = 8$ each group). Data were statistically analyzed by unpaired Student's t test ($^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with NS group).

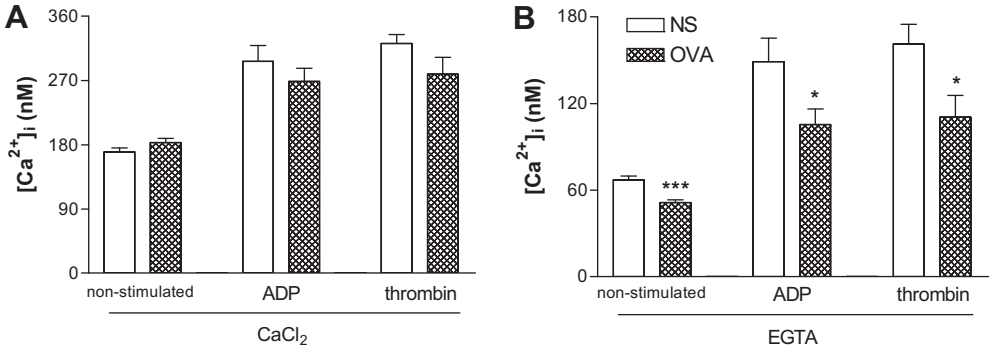


Fig. 5. Decrease in intracellular calcium levels in ovalbumin (OVA)-challenged rat platelets 24 h after challenge. Changes in total (panels A) and internal Ca^{2+} levels (panels B) in response to either ADP (20 μM) or thrombin (100 mU/ml) in platelets from non-sensitized (NS) and OVA-challenged rats. Internal Ca^{2+} levels were determined in Ca^{2+}-free medium and presence of EGTA (2 mM). Note that Y-axis in panel A varies from zero to 360 nM, whereas in panel B it varies from zero to 180 nM. Values are expressed as mean ± S.E.M. ($n = 8–10$ each group). Data were analyzed by unpaired Student's t test ($^{*}p < 0.05$; $^{***}p < 0.01$ compared with non-sensitized group).

[24]. Besides, plasma levels of β-thromboglobulin and platelet factor-4, markers of platelet activation *in vivo*, are elevated within 10 min after the onset of the asthmatic response to allergen in humans [25].

Platelet adhesion to the vessel wall (endothelial cells or components of the extracellular matrix such as fibrinogen) and subsequent spreading is reported to provide a sticky surface which promotes the recruitment of circulating leukocytes, which allows transendothelial migration of leukocytes [26–28]. Firm adhesion between leukocytes and platelets is mediated by direct interaction of leukocyte β2 integrins (LFA-1, Mac-1) with either platelet adhesion molecules (GPIbα, ICAM-2, functional adhesion molecule-3, JAM-A) or to bridging ligands on platelet receptors such as fibrinogen [29,30]. Platelet adhesion to extracellular matrix is a complex process that involves the interactions between components of the extracellular matrix and adhesion molecules expressed on the platelet surface. The platelet glycoprotein (GP) IIb/IIIa (integrin αIIβIII) receptor mediates platelet adhesion to matrix containing fibrinogen [31,32]. Thrombin and ADP agonists induce full platelet activation leading to conformational change of $\alpha_{IIb}\beta_3$ integrin from low-affinity state to high-affinity to fibrinogen molecule [33]. Our results showed that spontaneous platelet adhesion to fibrinogen was not affected by OVA-challenge in any studied time post-challenge. Although $\alpha_{IIb}\beta_3$ platelet integrin is able to bind to immobilized fibrinogen in unstimulated platelets [34], previous studies have shown that in allergic inflammation models, platelets participate in an activated rather than resting state [8,35]. This is consistent with our study showing that OVA-challenge potentiates platelet adhesion to immobilized fibrinogen only when stimulated with thrombin and ADP, with no modifications in platelet adhesion pattern in the absence of stimulus. In OVA-challenged rats, the ADP- and thrombin-induced platelet adhesion to immobilized fibrinogen was significantly increased at an immediate phase (30 min), but rather decreased at a late phase (24 h) after OVA-challenge. Of interest, platelet adhesion to cultured endothelial cells has been shown to depend upon the expression of $\alpha_{IIb}\beta_3$ (GP IIb/IIIa) on platelets and $\alpha_v\beta_3$ on endothelial cells [36,37], the latter of which is highly expressed in the endothelium of alveolar and extra-alveolar microvessels [38,39].

Thrombin interacts with PAR-1, PAR-3 or PAR-4 G-protein coupled receptors in the platelet surface leading to phospholipase C activation, thus hydrolyzing membrane phosphatidylinositol $4,5P_2$ to inositol triphosphate (IP_3), which in turn elevates intracellular calcium levels and activates protein kinase C (PKC). On the other hand, ADP acts on two receptors on platelets, namely the G_q-coupled $P2Y_1$ receptor and G_i-coupled $P2Y_{12}$ receptor to cause platelet activation [40]. $P2Y_1$ receptor stimulation results in the increase in intracellular calcium through the generation of IP_3 and activation of PKC whereas $P2Y_{12}$ receptor stimulation results in $G_{\alpha i}$-mediated inhibition of stimulated adenylate cyclase and $G_{\beta\gamma}$-mediated activation of phosphoinositide 3-kinase γ (PI3Kγ), protein kinase B (Akt/PKB) and Rap 1b. The Ca^{2+} influx in response to thrombin or ADP activates phospholipase A_2 that hydrolysate membrane phospholipids, enhancing the arachidonic acid availability and thromboxane A_2 synthesis, thus amplifying the primary responses of other agonists [41]. In our study, the increased platelet adhesion at 30 min post-OVA challenge was accompanied by significant rises in total Ca^{2+} influx and/or intracellular calcium levels. Nevertheless, the greater intracellular calcium levels was clearly more evident in conditions of absence of extracellular Ca^{2+} (omission of Ca^{2+} in Krebs solution and addition of EGTA), suggesting that amount of Ca^{2+} released from endoplasmic reticular to platelet cytosol is more critical for platelet adhesion, possibly as a consequence of activation of the IP_3 signaling pathway. A similar finding was reported in platelets of untreated asthmatic patients [35]. Elevation of IP_3 levels in platelets from asthmatics patients was also described [42]. Interestingly, the reduction of platelet adhesion at a late phase (24 h) post-OVA challenge was accompanied by lower intracellular calcium levels in platelets. It is likely therefore that antigen challenge causes an exhaustion of platelet activity at 24 h due to an early hyperactivity.

In conclusion, our study shows that allergen challenge lead to changes in ADP- and thrombin-stimulated platelets at immediate and late pulmonary allergic responses. Increased platelet adhesion to immobilized fibrinogen is accompanied to rises in cytosolic Ca^{2+} levels at an early time after OVA-challenge. Conversely, reduced platelet adhesion is observed at a late time after OVA-challenge that is accompanied by decreased intracellular calcium levels.

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