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NF-κB inhibitors impair platelet activation responses

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To cite this article: Malaver E, Romaniuk MA, D’Atri LP, Pozner RG, Negrotto S, Benzadón R, Schattner M. NF-κB inhibitors impair platelet activation responses. J Thromb Haemost 2009; 7: 1333–43.

See also Beaulieu LM, Freedman JE. NFκB regulation of platelet function: no nucleus, no genes, no problem? This issue, pp 1329–32.

Summary. Background: Although platelets are anucleated cells, they express several transcription factors that exert non-genomic functions, including the positive and negative regulation of platelet activation. NF-κB is a major transcriptional regulator of genes involved in survival, proliferation and inflammation. Objective: Because platelets play a critical role not only in hemostasis, but also in inflammation and tumor progression, we evaluated the role of NF-κB in platelet physiology. Results: Immunofluorescence, Western blotting and ELISA studies revealed that platelets express IκBα and NF-κB, and that stimulation with thrombin triggers IκBα phosphorylation and degradation and the binding of platelet NF-κB p65 subunit to synthetic oligonucleotides containing the consensus sequence for NF-κB. Two specific unrelated inhibitors of NF-κB activation, BAY 11-7082 and Ro 106-9920, reduced PAC-1 and fibrinogen binding to integrin αIIβ3 and restricted platelet spreading on immobilized fibrinogen. Both inhibitors impaired aggregation mediated by ADP, epinephrine, collagen or thrombin, but not arachidonic acid. ATP release, TXB2 formation, P-selectin expression, ERK phosphorylation and cPLA2 activity stimulated by thrombin were reduced in BAY 11-7082- or Ro 106-9920-treated platelets. Although bleeding time was not affected, ADP-induced platelet aggregation was impaired in mice treated with BAY 11-7082. Conclusions: Our results suggest that NF-κB may be a novel mediator of platelet responses. The blockade of platelet function by NF-κB inhibitors might be relevant in those clinical situations where these drugs are being considered for anti-tumor and/or anti-inflammatory therapy.

Keywords: cPLA2, NF-κB, platelets, TXB2, αIIβ3.

Introduction

The pleiotropic NF-κB normally exists as an inactive cytoplasmic complex whose predominant form is a heterodimer composed of p50 and p65 subunits tightly bound to inhibitory proteins of the IκB family [1]. Diverse stimuli, including cytokines, viral infection, UV radiation and free radicals, activate NF-κB through the phosphorylation of IκBα by the IKK complex. Phosphorylated IκBαs are rapidly polyubiquitinated and degraded by the proteasome. Following the release from its inhibitor, NF-κB dimers translocate to the nucleus, where they bind target genes and activate transcription. Genes regulated by NF-κB include those involved in inflammation, cell survival, differentiation and proliferation responses [1]. Thus, NF-κB is an attractive target for therapeutic intervention against cancer and inflammatory diseases.

Although platelets are anucleated cell fragments, recent reports show that platelets express transcription factors including the steroid/nuclear receptors [2], peroxisome proliferator activated receptor (PPAR) β/δ [3], PPARγ [4], the glucocorticoid receptor [5] and retinoid X receptors (RXR) [6]. The interaction of the estrogen receptor β with 17β-estradiol potentiates thrombin-induced platelet aggregation [7]. PPARγ agonists prevent platelet CD40L expression and the release of CD40L, TXB2 and ATP [4]. The binding of prednisolone to its receptor inhibits platelet aggregation [5]. RXR ligands inhibit aggregation and TXB2 release through the inhibition of protein Gq-induced Rac activation and intracellular Ca2+ release [6]. Together, these findings demonstrate that transcription factors can exert non-genomic functions on platelets. While it has been previously shown that IκBα is phosphorylated and degraded after platelet activation [8], the functional significance of the NF-κB/IκB complex was not investigated. Given the importance of both NF-κB and platelets in immune cell homeostasis, inflammation and tumor progression, we have investigated the role of NF-κB on platelet physiology.
Materials and methods

Reagents

ADP, arachidonic acid (AA), epinephrine, human α-thrombin, luciferin-luciferase, ATP and TRITC-phalloidin were from Sigma (St Louis, MO, USA). Collagen was from Nycomed Pharma (Unterschleibheim, Germany). The thrombin receptor Sigma (St Louis, MO, USA). Collagen was from Nycomed

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Immunofluorescence

WPs and leukocytes were fixed with 1% paraformaldehyde and cytospined. Cells were treated with PBS/0.1% Triton, blocked and incubated with or without (negative controls) rabbit polyclonal-anti-NF-κB or mouse monoclonal-anti-NF-κB in combination with mouse or rabbit-anti-vWF, respectively. After washing, cells were stained with FITC-anti-rabbit and Cy3-anti-mouse secondary Abs, mounted with Vectashield and visualized under fluorescent microscopy.

Immunoblotting

WPs lysates (1–2 × 10^7 cells/mL) were prepared by solubilizing platelets in loading buffer (62.5 mM Tris–HCl at pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol) in the presence of a protease inhibitor cocktail containing 1 mM AEBSF, 0.008 mM Aprotinin, 0.02 mM Leupeptin, 0.04 mM Bestatin, 0.015 mM Pepstatin A and 0.014 mM E-64 (Sigma). Equal amounts of proteins were electrophoresed on a 12% SDS-PAGE and electro-transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking, the membranes were incubated overnight at 4 °C with primary Abs followed by a HRP secondary Ab. Protein bands were visualized by using the ECL reaction. Immunoblotting results were quantitated using GEL-PRO analyzer 3.1 software and values from blot reprobes were used for normalization of data for protein loads.

NF-κB ELISA assay

NF-κB DNA binding ability was measured by using an ELISA kit from Panomics (Fremont, CA, USA). In this method, activated NF-κB from cell lysates binds to a NF-κB p65 consensus binding site on a biotinylated oligonucleotide. These oligonucleotides are then immobilized on a streptavidin-coated plate. The bound NF-κB p65 is detected by a specific Ab. An additional HRP-secondary Ab provides a sensitive colorimetric readout that is quantified by spectrophotometry.

Flow cytometry

WPs were treated with the inhibitors, stimulated, fixed and incubated in PBS/0.1% FBS/CD62P or isotype FITC-IgG1. Flow cytometry analysis was performed on a FACSCalibur flow cytometer® using CELLQUEST software (BD Biosciences). A similar technique was employed to evaluate α₄β₁ integrin activation or fibrinogen binding by using FITC-PAC-1 or Alexa-488 fibrinogen, respectively.

Ca^{2+} levels were determined by loading PRP for 30 min at 37 °C with fluo-3 AM (2 μM). After centrifugation platelets were resuspended in Tyrode’s buffer. To determine the amount of platelets that underwent an increase in intracellular Ca^{2+}, a threshold value was determined for each experiment in a time gate (15 s) before the addition of thrombin. Then, changes in intracellular Ca^{2+} were measured for 1 min after stimulation. Results were expressed as % of positive cells and represent the events with FL1 values above the threshold.

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Platelet spreading

After incubation with NF-κB inhibitors, WPs (5 × 10^7/mL) were stimulated and plated on fibrinogen (150 μg/mL)-coated slides. Adhered platelets were fixed, permeabilized, stained with TRITC-Phalloidin, mounted and visualized under fluorescent microscopy.

Platelet aggregation and ATP release

Aggregation and ATP release were measured in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA). ATP levels were measured at the end of the assay by adding a known amount of standard ATP (2 μM).

Measurement of TXB2 release

WPs were incubated for 5 min in a platelet aggregometer stirring at 1000 rpm with thrombin or AA. The reaction was stopped by the addition of ice-cold PBS/2 mM EDTA/500 μM aspirin. Samples were centrifuged, and TXB2 levels in the supernatants were measured using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

Platelet cytosolic phospholipase A2 (cPLA2) activity assay

WPs stimulated with thrombin were centrifuged and sonicated in ice-cold HEPES buffer (50 mM HEPES, 1 mM EDTA, pH 7.4) containing a protease inhibitor cocktail. After centrifugation (10 000 g, 15 min, 4 °C), the supernatants were stored at −80 °C until use. cPLA2 activity was measured using a kit from Cayman Chemical.

Mice studies

Male BALB/c mice (18–22 g; between 8 and 10 weeks of age) were housed and bred in the animal facility at the Hematological Research Institute under the Argentine Home Office regulations for the care and use of animals. BAY 11-7082 (10 mg/kg) or vehicle was administered intraperitoneally. After 24 h the same dose was repeated and 2 h later the bleeding time was measured and blood was collected by puncture of the retro-orbital plexus.

Bleeding time

Mice were anesthetized with pentobarbital, and the tail was cut 5 mm from the distal tip and immersed in PBS at 37 °C. The bleeding time was defined as the time point at which all visible signs of bleeding from the incision had stopped.

Preparation of murine PRP

Whole blood was diluted 1:1 in PBS containing 1 mM Ca^{2+} and Mg^{2+} and centrifuged. The platelet count was adjusted to 3 × 10^8/mL with platelet poor plasma. Aggregation was measured as described for human platelets.

Statistical analysis

Results are expressed as means ± SEM. The Student’s paired t-test and the Mann–Whitney U-test (for mice experiments) were employed to determine the significance of differences between the groups. A P value lower than 0.05 was considered to be statistically significant.

Results

Platelets contain NF-κB and platelet activation triggers IκBα phosphorylation and degradation and NF-κB release

Immunofluorescence (Fig. 1A) and Western blot studies (Fig. 1B, lane 1) show that human platelets express NF-κB. To rule out the possibility that the Western blot signal was due to leukocyte contamination, WPs were further purified by using a high-efficiency leukoreduction filter (see Materials and methods). Fig. 1(B) (lanes 4–7) shows that two different Abs directed against human NF-κB revealed a clear p65 band in leukocyte-free platelet preparations from four different donors. In addition, no signal was observed when p65 protein was analyzed using the same number of leukocytes present in WPs preparations, while a clear band was detected with higher leukocyte numbers (Fig. 1B, lanes 2 and 3).

In nucleated cells, the simplest model of NF-κB activation implies phosphorylation, ubiquitination and degradation of its inhibitor followed by binding of the released NF-κB dimers to their consensus DNA sequences [1]. Fig. 2(A–C) shows that the activation of leukocyte-free WPs with thrombin resulted in phosphorylation (Fig. 2A) and more than a 50% degradation of IκBα (Fig. 2B). Both events were prevented by pretreating the platelets with BAY 11-7082, a specific inhibitor of NF-κB phosphorylation [9]. Interestingly, Ro 106-9920, another NF-κB inhibitor non-structurally related to BAY 11-7082, which selectively inhibits IκBα ubiquitination [10], showed a similar inhibitory activity against IκBα degradation without modifying its phosphorylation (Fig. 2A–C).

Furthermore, ELISA assays showed that NF-κB p65 from leukocyte-free thrombin-stimulated WP lysates bound to oligonucleotides containing the DNA consensus sequence for NF-κB (Fig. 2D). This DNA binding ability was also observed in lysates from platelets stimulated with PAR1-AP and PAR4-AP, ADP, AA or collagen. Preincubation of platelets with BAY 11-7082 abrogated these responses (Fig. 2D).

Inhibition of PAC-1 binding, fibrinogen binding and platelet spreading by BAY 11-7082 and Ro 106-9920

To determine the role of NF-κB in platelet physiology, we examined functional responses in the presence of BAY 11-7082 or Ro 106-9920. As a consequence of the platelet-agonist binding...
interaction, αIIbβ3 undergoes conformational changes allowing the exposure of its high-affinity fibrinogen-binding site (inside-out signaling); one of the resulting neoepitopes is recognized by the monoclonal Ab PAC-1. Fig. 3(A,B) shows that both thrombin-mediated PAC-1 and soluble fibrinogen binding were significantly decreased in BAY 11-7082 or Ro106-9920-treated platelets, as compared with control samples.

Fibrinogen binding results in postligand occupancy events (outside-in signaling) that lead to platelet shape change and spreading [11]. In agreement with the reduced PAC-1 and fibrinogen binding, pretreatment of platelets with the NF-κB inhibitors resulted in a marked decrease of thrombin-induced adhesion, filopodia formation and spreading of platelets (Fig. 3C).

The increase of cytosolic Ca²⁺ levels is a key early event that follows platelet stimulation [12]. Interestingly, NF-κB appears not to influence Ca²⁺ mobilization because BAY 11-7082– or Ro 106-9920-treated platelets and control platelets demonstrated an equivalent ability to increase intracellular Ca²⁺ (Fig. 3D).

### Effect of NF-κB inhibitors on platelet aggregation

Figure 4 shows that WPs aggregation mediated by low thrombin concentration was markedly inhibited by BAY 11-7082 or Ro 106-9920. The decrease in PAR1-AP- or PAR4-AP-mediated aggregation by these drugs suggests that the signaling pathways triggered by both thrombin receptors are influenced by the NF-κB inhibitors.

To analyze whether the inhibition mediated by these compounds was also observed in a more physiologic environment and whether it was specific for thrombin, aliquots of PRP were stimulated with agonists that act through different mechanisms. Treatment of PRP with both NF-κB inhibitors significantly inhibited the aggregation induced by ADP, epinephrine or collagen (Fig. 5). In contrast, the aggregation response induced by AA was not modified, even when the inhibitors were added at a concentration of 100 μM. In all cases, not only was the secondary phase completely abolished, but the initial ADP- and epinephrine-induced aggregation was also significantly impaired. Increasing the concentrations of the

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**Fig. 1.** Platelets express NF-κB. (A) Double immunofluorescence microscopy of resting platelets using rabbit or mouse anti-NF-κB and mouse or rabbit anti-vWF primary Abs followed by anti-rabbit-FITC/anti-mouse-Cy3 immunoglobulins. Leukocytes were stained using rabbit or mouse anti-NF-κB followed by anti-rabbit-FITC/anti-mouse-Cy3 immunoglobulins. Nuclei were counterstained with DAPI. Images are representative of three independent experiments. (B) WPs, leukocyte-free WPs (see Materials and methods) or leukocyte lysates were immunoblotted with polyclonal or monoclonal anti-NF-κB p65 Ab or with an anti-actin Ab to indicate comparable loading levels. Lane 1, WPs; lane 2, leukocytes (similar number as in the WPs sample from lane 1); lane 3, leukocytes; lanes 4–7, leukocyte-free WPs from different donor samples.
agonists bypassed the inhibitory action of both inhibitors (data not shown).

Effect of BAY 11-7082 and Ro 106-9920 on TXB<sub>2</sub> formation, ATP release and P-selectin expression

The secondary wave of aggregation induced by most agonists is mainly dependent on TXA<sub>2</sub> production and ADP release from dense granules. To analyze whether the inhibitory activities of BAY 11-7082 and Ro 106-9920 were related to the impairment of these responses, we estimated the levels of TXA<sub>2</sub> by measuring its stable metabolite, TXB<sub>2</sub>, and measured ATP release as an indicator of dense granule secretion. Fig. 6(A) shows that the TXB<sub>2</sub> formation triggered by thrombin was significantly decreased in platelets that were preincubated with either BAY 11-7082 or Ro 106-9920. However, these inhibitors did not affect the conversion of exogenous AA into TXB<sub>2</sub>. Similar results were obtained in the ATP assay (Fig. 6B). There was no ATP release in BAY 11-7082- or Ro 106-9920-treated platelets stimulated with low thrombin concentrations, while there was normal ATP release in AA-activated cells. In addition, not only dense, but also alpha granule secretion appeared to be influenced by the NF-κB inhibitors, because flow cytometry studies showed that P-selectin expression triggered by thrombin (90 ± 5% of positive cells) was reduced in BAY 11-7082- or Ro 106-9920-treated platelets (18 ± 4% and 20 ± 2% respectively; n = 4; P < 0.01 vs. thrombin).

Inhibition of platelet cPLA<sub>2</sub> activity and ERK phosphorylation by NF-κB inhibitors

The observation that NF-κB inhibitors suppressed the aggregation induced by low collagen or thrombin concentrations, as well as the secondary aggregation triggered by ADP and epinephrine, but had no effect on similar platelet responses induced by AA, suggested that the endogenous AA release could be a target for these drugs. To evaluate whether the inhibitory action was associated with an alteration in the main enzyme that accounts for the AA release, we analyzed cPLA<sub>2</sub> activity. Treatment of platelets with BAY 11-7082 or Ro 106-9920 significantly reduced the thrombin-induced enzyme activity (Fig. 7A).
It has been reported that upon stimulation, ERK2 is activated in human platelets [13]. In this context, phosphorylated ERK2 mediates the phosphorylation of cPLA2 and increases its activity [14]. We found that thrombin-triggered ERK2 phosphorylation was markedly inhibited by BAY 11-7082 or Ro 106-9920 (Fig. 7B).

Administration of BAY 11-7082 impairs in vitro platelet aggregation

To determine the relevance of NF-κB inhibition in vivo, we evaluated the hemostatic response in mice treated with BAY 11-7082 and we found no differences in the bleeding time between treated and untreated groups. However, in vitro platelet aggregation was impaired in those animals that were treated with BAY 11-7082 (Fig. 8).

Discussion

Our results show that NF-κB is expressed in platelets and that platelet stimulation with different stimuli results in both the phosphorylation/degradation of the NF-κB inhibitor and the ability of NF-κB in platelet extracts to bind to DNA. Moreover, BAY 11-7082 and Ro 106-9920, two chemically unrelated NF-κB inhibitors, suppressed different platelet responses, such as cellular spreading, the inside-out signaling...
pathway of αIIbβ3 and ADP- and epinephrine-induced primary aggregation. These observations suggest that NF-κB is involved in the regulation of the initial stages of platelet activation, including the cytoskeletal rearrangements that lead to platelet shape change and the active form of the αIIbβ3 integrin. Although BAY 11-7082 and Ro 106-9920 suppressed the aggregation response mediated by ADP, epinephrine and low concentrations of collagen or thrombin, they failed to impair the AA-induced response. These findings indicate that the inhibitory activity of these drugs is not related to a blockade of the cyclooxygenase enzyme. Activation of cPLA2 is regulated by both an increase in intracellular Ca^{2+} levels [15] and by the phosphorylation of p38 or ERK2 [14,16]. Surprisingly, the intracellular Ca^{2+} increase following

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thrombin stimulation was not modified by the NF-κB inhibitors. However, because flow cytometry does not quantify differences in Ca^{2+} concentration, and only changes in the late, sustained calcium level after stimulation can be observed, further studies are required to precisely define the effect of these drugs on Ca^{2+} levels. Interestingly, thrombin-mediated ERK
phosphorylation was markedly inhibited, suggesting that decreased cPLA2 phosphorylation by ERK might account for the diminished enzyme activity. A similar pattern of altered platelet responses was recently described in a patient with an inherited cPLA2 deficiency [17]. It has also been demonstrated that a pool of cPLA2 is associated with αIIbβ3, and that the activity of cPLA2 increases after fibrinogen binding [18]. Because we have also observed that NF-κB inhibitors decreased PAC-1, fibrinogen binding and spreading, a reduced αIIbβ3 outside-in/inside-out signaling could contribute to the reduced cPLA2 activity in BAY 11-7082 and Ro 106-9920-treated platelets in addition to the reduced phosphorylation of cPLA2. Because we observed that the degradation of IkBz, the release of NF-κB molecules and the inhibition of NF-κB by BAY 11-7082 and Ro 106-9920 are rapid events, any resulting effect of these phenomena most likely involves protein–protein interactions [3,4,6]. Given that cPLA2 interacts with αIIbβ3 [18] and that NF-κB inhibitors decrease cPLA2 activity, it could be speculated that the modulation of NF-κB by platelet agonists promotes the formation of a complex that includes αIIbβ3-NF-κB-ERK-cPLA2. In this context, it has been shown that the αIIbβ3 receptors acquired by neutrophils through platelet-derived microparticles cooperate with β2 integrins to activate NF-κB signaling [19]. In addition, activation of other transcription factors like estrogen receptor, sensitizes platelets to agonists by promoting the Src kinase-dependent activation of αIIbβ3 [7].

Platelets are widely recognized as key mediators of inflammatory responses. The expression of P-selectin on the membrane of activated platelets is the main link between platelets and inflammatory cells [20]. Interestingly, both inhibitors decreased P-selectin expression, suggesting that the non-genomic role of NF-κB in platelets could be associated with the regulation of both hemostatic- and inflammatory-mediated responses. Finally, our data demonstrating that ADP-induced aggregation was inhibited in mice treated with BAY 11-7082 suggest that the platelet inhibition due to NF-κB suppression might also be relevant in vivo.

In conclusion, we have presented evidence that: (i) NF-κB is expressed in platelets; (ii) platelet agonists trigger IkBz phosphorylation/degradation and the binding of platelet NF-κB to DNA; (iii) two unrelated specific NF-κB inhibitors are capable of negatively regulating platelet spreading, aggregation and granule release through the blockade of the ERK-cPLA2-
TXA2 pathway; and (iv) treatment of mice with BAY 11-7082 inhibits platelet aggregation. Together, these results suggest that NF-κB could be a mediator of platelet function. Nevertheless, a usual concern about the use of pharmacological inhibitors is their potential side effects. It has been demonstrated that BAY 11-7082 does not act as a global inhibitor of cytokine-mediated phosphorylation, but selectively inhibits the phosphorylation of IκBα [9]. In addition, Ro 106-9920, despite not being structurally related to BAY 11-7082, selectively inhibits IκBα by interfering with its ubiquitination, but not its phosphorylation [10]. In fact we have evaluated platelet IκBα phosphorylation induced by thrombin in the presence of both inhibitors and as expected, BAY 11-7082 but not Ro 106-9920 inhibited IκBα phosphorylation in a concentration-dependent manner, strengthening the notion that the two drugs have different mechanisms of action. Thus, although it is highly conceivable that the inhibitory effect of these drugs on platelet function is due to a selective inhibition of the NF-κB pathway, we do not rule out the possibility that these compounds may have alternative modes of action.

The constitutive activation of NF-κB is a hallmark of many malignant tumors and accounts for profound chemoresistance. Therefore, the inhibition of NF-κB activation has been shown to be a useful strategy for increasing the sensitivity of cytostatic drug treatment. Moreover, targeting NF-κB signaling is also emerging as a potential therapy to prevent restenosis after percutaneous coronary intervention [21] and hypertension [22]. Because platelet activation is not only linked to hemostasis, but also has a relevant role in inflammation and metastasis, our present data demonstrating that NF-κB inhibitors interfere with platelet function may have a great impact when these types of drugs are considered for the treatment of cancer and various inflammatory diseases.

Acknowledgements

The authors are grateful to the CEMIC technicians for collecting the blood samples. This work was supported by grants from ANPCYT (PICTs 1990 and 25754).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol 2008; 8: 837–48.
2 Khetawat G, Faraday N, Nealen ML, Vijayan KV, Bolton E, Noga SJ, Bray PF. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood* 2000; 95: 2289–96.

3 Ali FY, Davidson SJ, Moraes LA, Traves SL, Paul-Clark M, Bishop-Bailey D, Warner TD, Mitchell JA. Role of nuclear receptor signaling in platelets: antithrombotic effects of PPARbeta. *FASEB J* 2006; 20: 326–8.

4 Akbiyik F, Ray DM, Gettings KF, Blumberg N, Francis CW, Phipps RP. Human bone marrow megakaryocytes and platelets express PPARgamma, and PPARgamma agonists blunt platelet release of CD40 ligand and thromboxanes. *Blood* 2004; 104: 1361–8.

5 Moraes LA, Paul-Clark MJ, Rickman A, Flower RJ, Goulding NJ, Perretti M. Ligand-specific glucocorticoid receptor activation in human platelets. *Blood* 2005; 106: 4167–75.

6 Moraes LA, Swales KE, Wray JA, Damazo A, Gibbins JM, Warner TD, Bishop-Bailey D. Nongenomic signaling of the retinoid X receptor through binding and inhibiting Gq in human platelets. *Blood* 2007; 109: 3741–4.

7 Moro L, Reineri S, Piranda D, Pietrapiana D, Lova P, Bertoni A, Graziani A, Delfilippi P, Canobbio I, Torri M, Sinigaglia F. Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase. *Blood* 2005; 105: 115–21.

8 Liu F, Morris S, Epps J, Carroll R. Demonstration of an activation regulated NF-kappaB/1-kappaBalpha complex in human platelets. *Thromb Res* 2002; 106: 199–203.

9 Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* 1997; 272: 21096–103.

10 Swinney DC, Xu YZ, Scarfia LE, Lee I, Mak AY, Gan QF, Ramesha CS, Mulkins MA, Dunn J, So OY, Biegel T, Dinh M, Volkel P, Barnett J, Dalrymple SA, Lee S, Huber M. A small molecule ubiquitination inhibitor blocks NF-kappaB-dependent cytokine expression in cells and rats. *J Biol Chem* 2002; 277: 23573–81.

11 Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 2004; 104: 1606–15.

12 Sage SO. The Wellcome Prize Lecture. Calcium entry mechanisms in human platelets. *Exp Physiol* 1997; 82: 807–23.

13 Adam F, Kauskot A, Rosa JP, Bryckaert M. Mitogen-activated protein kinases in hemostasis and thrombosis. *J Thromb Haemost* 2008; 6: 2007–16.

14 Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. *J Thromb Haemost* 2006; 4: 638–47.

15 Leslie CC. Properties and regulation of cytosolic phospholipase A2. *J Biol Chem* 1997; 272: 16709–12.

16 Borsch-Haubold AG, Kramer RM, Watson SP. Phosphorylation and activation of cytosolic phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. *Eur J Biochem* 1997; 245: 751–9.

17 Adler DH, Cogan JD, Phillips JA 3rd, Schnez-Boutaud N, Milne GL, Iverson T, Stein JA, Brenner DA, Morrow JD, Boutaud O, Oates JA. Inherited human cPLA2alpha deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. *J Clin Invest* 2008; 118: 2121–31.

18 Prevost N, Mitisos JV, Kato H, Burke JE, Dennis EA, Shimizu T, Shattil SJ. Group IVA cytosolic phospholipase A2 (cPLA2alpha) and integrin alphaIIbbeta3 reinforce each other’s functions during alphaIIbbeta3 signaling in platelets. *Blood* 2009; 113: 447–57.

19 Salanova B, Choi M, Rolle S, Wellner M, Luft FC, Kettritz R. Beta2-integrins and acquired glycoprotein IIb/IIIa (GPIIb/IIIa) receptors cooperate in NF-kappaB activation of human neutrophils. *J Biol Chem* 2007; 282: 27960–9.

20 Zarbock A, Polansawksa-Grabowska RK, Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev* 2007; 21: 99–111.

21 Egashira K, Suzuki J, Ito H, Aoki M, Isobe M, Morishita R. Long-term follow up of initial clinical cases with NF-kappaB decoy oligodeoxynucleotide transfection at the site of coronary stenting. *J Gene Med* 2008; 10: 805–9.

22 Li XC, Zhuo JL. Nuclear factor-kappaB as a hormonal intracellular signaling molecule: focus on angiotensin II-induced cardiovascular and renal injury. *Curr Opin Nephrol Hypertens* 2008; 17: 37–43.