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The NO/cGMP pathway inhibits Rap1 activation in human platelets via cGMP-dependent protein kinase I

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Summary

The NO/cGMP signalling pathway strongly inhibits agonist-induced platelet aggregation. However, the molecular mechanisms involved are not completely defined. We have studied NO/cGMP effects on the activity of Rap1, an abundant guanine-nucleotide-binding protein in platelets. Rap1-GTP levels were reduced by NO-donors and activators of NO-sensitive soluble guanylyl cyclase. Four lines of evidence suggest that NO/cGMP effects are mediated by cGMP-dependent protein kinase (cGKI): (i) Rap1 inhibition correlated with cGKI activity as measured by the phosphorylation state of VASP, an established substrate of cGKI, (ii) 8-pCPT-cGMP, a membrane permeable cGMP-analog and activator of cGKI, completely blocked Rap1 activation, (iii) Rp-8pCPT-cGMPS, a cGKI inhibitor, reversed NO effects and (iv) expression of cGKI in cGKI-deficient megakaryocytes inhibited Rap1 activation. NO/cGMP/cGKI effects were independent of the type of stimulus used for Rap1 activation. Thrombin-, ADP- and collagen-induced formation of Rap1-GTP in platelets as well as turbulence-induced Rap1 activation in megakaryocytes were inhibited. Furthermore, cGKI inhibited ADP-induced Rap1 activation induced by the G_i-coupled P2Y₁₂ receptor alone, i.e. independently of effects on Ca²⁺-signalling. From these studies we conclude that NO/cGMP inhibit Rap1 activation in human platelets and that this effect is mediated by cGKI. Since Rap1 controls the function of integrin $\alpha_{IIb}\beta_3$, we propose that Rap1 inhibition might play a central role in the anti-aggregatory actions of NO/cGMP.

Introduction

Platelet aggregation is a crucial event in the development of arterial thrombosis and is mediated by the binding of fibrinogen to activated integrin $\alpha_{IIb}\beta_3$ receptors on the surface of platelets. Platelet activation and aggregation can be blocked by nitric oxide (NO) (1, 2). NO is derived either from endothelial cells or platelets (3) and activates soluble NO-sensitive guanylyl cyclase (sGC) resulting in the production of cGMP (4). The main targets of cGMP in platelets are cGMP-dependent protein kinase I α (cGKI) as well as cGMP-regulated PDEs 2, 3 and 5 (5, 6). Data from human (7) and mouse (8) cGKI-deficient platelets suggest that cGKI is an important mediator of NO/cGMP effects in platelets. For example, *in vivo* studies of the microcirculation after ischemia/reperfusion in cGKI-deficient mice clearly showed that cGKI prevents intravascular platelet aggregation (8).

Recently, the small guanine-nucleotide-binding protein Rap1 has attracted a lot of interest as major regulator of integrin activity (9-12). Rap1A and B isoforms have been described exhibiting 95 % sequence identity and platelets express particularly high levels of Rap1B (13-15). Rap1 efficiently activates various integrins including the platelet integrin $\alpha_{IIb}\beta_3$, which mediates fibrinogen binding required for platelet aggregation (16, 17). Very recently, deletion of mouse CalDAG-GEFI (also known as RasGRP2), a guanine-nucleotide-exchange factor (GEF) involved in the activation of Rap1, was shown to result in reduced platelet aggregation and increased bleeding time (18). Rap1 is a common target of many different activation pathways in platelets. ADP, thrombin, collagen, epinephrine and platelet-activating factor induce Rap1-GTP formation (19). The pathways leading to Rap1 activation in platelets are controversial. For example, ADP has been suggested to activate Rap1 by Ca^{2+} -dependent (19, 20) as well as Ca^{2+} -independent mechanisms involving the G_{β_i} -coupled P2Y₁₂ receptor (21, 22). Recently, phosphatidylinositol 3-kinase (PI3K) was reported to mediate Rap1 activation by different platelet agonists (22-24). In megakaryocytes (platelet progenitors) turbulence

activates Rap1 by a yet undefined mechanism independent of PLC, PKC, Ca²⁺, PI3K and actin dynamics (17).

Rap1A and B are known to be phosphorylated by cyclic nucleotide-regulated kinases and the platelet isoform Rap1B was reported to be the preferred substrate for these kinases (13, 25, 26). However, Rap1B phosphorylation has a slow time course and does not affect GTP-binding or GTPase activity of Rap1 (25, 27). In the present study we have investigated possible effects of NO on stimulus-induced Rap1 activation. We show that NO inhibits Rap1-GTP formation in human platelets. Since Rap1 is an important regulator of integrin function and platelet aggregation, we suggest that NO-induced Rap1 inhibition might be responsible for the strong anti-aggregatory function of NO. Furthermore, we show that NO effects on Rap1 involve cGMP as well as cGKI. We also provide initial data on the possible mechanisms involved in Rap1 inhibition.

Materials and Methods

Reagents

Human thrombin, ADP, rat collagen I, YC-1, ODQ and MRS2179 were obtained from Sigma. DEA-NOate and DETA-NOate were purchased from ALEXIS Biochemicals. 8pCPT-cGMP and Rp-8pCPT-cGMPS were from Biolog. Polyclonal antibody against Rap1 (sc-65) was from Santa Cruz Biotechnology. Antibodies against P-VASP serine 239 (16C2) and cGKI have been described previously (28, 29). HRP-coupled goat-anti-rabbit and goat-anti-mouse were from Dianova. GSH Sepharose 4B was from Amersham Biosciences. cDNA for the GST-Rap-binding domain (RBD) of the Ral guanine nucleotide dissociation stimulator was kindly provided by Johannes L. Bos (Utrecht).

Platelet preparation

20.8 ml freshly drawn venous blood from healthy volunteers, who gave their informed consent according to the declaration of Helsinki, was collected into 4.2 ml prewarmed ACD buffer (85 mM citric acid, 65 mM sodium citrat, 100 mM glucose) and centrifuged at 200 g and RT for 15 min without breaks. Platelet rich plasma (PRP) was recovered and platelets were pelleted at 500 g and RT for 10 min and carefully resuspended in resuspension buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7,4) to a final concentration of 2x10⁸ platelets/ml. The platelet suspension was stored at RT for 30 min previous to experiments.

Infection of MEG-01 cells

MEG-01 megakaryocytes were obtained from ATCC and cultured in RPMI1640 medium supplemented with 20% heat-inactivated fetal calf serum and penicillin and streptomycin. cDNAs for wild-type cGKI□ or a catalytically inactive mutant, cGKI□-K405A under control of a CMV-promoter, were transfected into MEG-01 cells using adenoviral vectors as described (29). For infection cells were incubated with 1x10¹⁰ virus particles/ml for 2 hours in serum-free medium followed by addition of complete medium for 24 hours.

Rap1-GTP assay in platelets and MEG-01 cells

Washed platelets (aliquots of 500 μl) were equilibrated to 37°C in a waterbath for 10 min followed by preincubation with NO-donors DEA-NOate and DETA-NOate for 1 min, 10 or 30 μM YC-1 for 10 min, 30 μM ODQ for 30 min, indicated concentrations of 8pCPT-cGMP for 10 min, 200 μM Rp-8pCPT-cGMPS for 15 min and 50 μM MRS2179 for 1 min at 37°C. Then platelets were activated with thrombin (1 U/ml), ADP (10 μM) and collagen I (50 μg/ml) for the indicated times. Stimulations were terminated by adding 500 μl of ice-cold 2-fold lysis buffer (final concentrations of 1% (v/v) NP-40, 10% (v/v) glycerol, 50 mM Tris-

HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl₂, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin).

Rap1-GTP assays in megakaryocytes were performed two days after adenoviral infection. 24 hours before the assay MEG-01 cells were incubated overnight in serum-free medium. The next day cells were resuspended at 20x10⁶ cells/ml in medium. 500 μl aliquots of cell suspension in 1.5 ml tubes were incubated for 30 min at 37°C, then samples were mixed by inverting tubes 5 times up and down followed by 2 min rest at 37°C. Reactions were terminated by adding 500 μl of 2-fold lysis buffer as described above.

Platelets and MEG-01 cells were lysed and insoluble material was pelleted. Samples of 50 μl were removed for analysis of total Rap1, cGKI and phosphorylated VASP. 75 μl of a 10% (v/v) glutathione sepharose beads suspension saturated with RalGDS-RBD were added to the supernatant and incubated at 4°C for 45 min. Beads were washed four times in 1x lysis buffer and finally boiled in 3x SDS sample buffer. Samples were analyzed by Western blot using nitrocellulose membranes (Schleicher & Schuell) and antibodies against Rap1, cGKI and phosphorylated VASP followed by HRP-coupled secondary antibodies and enhanced chemiluminescence detection (Amersham). All experiments shown were performed at least three times with similar results.

Results

NO inhibits Rap1 activation

To characterize the effects of NO/cGMP on Rap1 activity we used a pull-down assay specific for active Rap1 developed by the group of J. Bos (19). In this assay the Ras binding domain of RalGDS fused to GST is used as a specific probe for active Rap1-GTP. In our studies we used washed platelets and Rap1 activation was induced by thrombin, ADP and collagen. NO effects depend, at least in part, on the type of NO-donor used (30). For this reason we chose

two NONOate molecules with different NO liberation properties as NO-donors: DEA-NO, which has a half-life of 2 min at 37°C and the slow-releasing substance DETA-NO ($t_{1/2}=20\text{h}$). DEA-NO (Fig. 1A) as well as DETA-NO (not shown) inhibited thrombin-induced formation of Rap1-GTP. Furthermore, ADP- and collagen-induced Rap1 activation were inhibited by NO-donors (Figs. 1B, C and Fig. 4). Treatment of platelets with DETA-NO alone had no effect on basal Rap1 activity (Fig. 1B, lanes 2-4).

NO effects are mediated by cGMP

An important target of NO in platelets is the cGMP generating enzyme sGC. To verify a role for sGC in NO-induced Rap1 inhibition we used YC-1, an NO-independent activator of sGC (4). YC-1 inhibited thrombin-, ADP- (Fig. 2A and 2B) and collagen-induced (not shown) Rap1 activation. Furthermore blocking of sGC with ODQ, an inhibitor of sGC (31), abolished NO effects on collagen-induced Rap1 activation (compare middle lane with last lane in Fig. 2C). In parallel we analyzed the phosphorylation state of VASP, an actin and focal adhesion binding protein and established substrate of cGKI in platelets (28, 29). For this purpose we used a previously described phosphorylation specific antibody (28). YC-1- and DETA-induced reduction of Rap1-GTP levels correlated with increased VASP phosphorylation, i.e. increased cGKI activity (Fig. 2).

cGMP effects are mediated by cGKI

To further evaluate if cGKI can mediate cGMP effects on Rap1 activity we used the membrane-permeable cGMP-analog 8pCPT-cGMP. Increasing concentrations of 8-pCPT-cGMP strongly reduced Rap1-GTP levels independently of the platelet agonists used for Rap1 activation including thrombin, ADP and collagen I (Fig. 3). As shown before in Fig. 2 activation of cGKI also resulted in the phosphorylation of VASP and the amount of VASP phosphorylation correlated with the decrease in Rap1-GTP.

To provide further evidence that cGKI was mediating NO effects we inhibited cGKI function with Rp-8pCPT-cGMPS in the presence of the NO-donor DEA-NO. As shown before (Fig. 1) DEA-NO reduced Rap1-GTP levels (Fig. 4, middle lanes). The cGKI inhibitor Rp-8pCPT-cGMPS abrogated NO effects indicating that cGKI was involved in NO-induced Rap1 inhibition (Fig. 4, right lanes). Rp-8pCPT-cGMPS alone did not affect ADP- and thrombin-induced formation of Rap1-GTP (data not shown).

cGKI inhibits Ca²⁺-independent activation of Rap1

NO/cGMP/cGKI is known to inhibit platelet signalling by inhibition of Ca²⁺ release from intracellular stores (32). Also, the Ca²⁺-dependent Rap1-GEF CalDAG-GEFI/RasGRP2 has been found in mouse platelets (18). Therefore we investigated if cGMP effects could be explained by the inhibition of Ca²⁺ mobilization. Rap1 activation by ADP has been reported to involve the G_q-coupled P2Y₁ receptor inducing release of Ca²⁺ from intracellular stores as well as the Ca²⁺-independent G_i-coupled P2Y₁₂ receptor (33). First we blocked G_q-mediated signalling with MRS2179, a specific inhibitor of the P2Y₁ receptor (34). Increasing concentrations of MRS2179 diminished ADP-induced Rap1 activation and the maximal effect was reached with 30 μM MRS2179 (Fig. 5A). This concentration of MRS2179 corresponds to the amount necessary to block mobilization of intracellular Ca²⁺ stores in platelets completely (34). Consequently, the remaining ADP-induced Rap1 activation is mediated exclusively by G_i-coupled pathways. Addition of the cGKI activator 8-pCPT-cGMP in the presence of MRS2179 further reduced the level of Rap1-GTP (Fig. 5B and 5C). From these experiments we conclude that Rap1 activation in platelets requires Ca²⁺-dependent as well as Ca²⁺-independent signals. Secondly, cGKI can inhibit G_i-mediated Ca²⁺-independent Rap1 activation.

cGKI inhibits turbulence-induced Rap1 activation in megakaryocytes

To gain further insight into the mechanisms involved in NO/cGMP/cGKI-induced Rap1 inhibition we studied Rap1 regulation in megakaryocytes, i.e platelet progenitor cells. Rap1 has been shown to be activated by turbulence in MEG-01 megakaryocytes (17). Rap1 activation occurred independently of PLC-, PKC-, Ca²⁺- and PI3K-signalling as well as actin dynamics in these cells (17). Because MEG-01 cells do not express detectable amounts of endogenous cGKI (Fig. 6, middle), we introduced wild-type cGKI as well as a catalytically inactive control mutant into MEG-01 cells using adenoviral vectors. The inactive cGKI bears a point mutation of an essential lysine in the catalytic site (29). Wildtype cGKI inhibited turbulence-induced Rap1 activation in MEG-01 cells (Fig. 6). The catalytically inactive mutant cGKI was incapable of mediating this effect.

Discussion

In the present study we show that Rap1 activation in human platelets is regulated by NO via cGMP and cGKI. Different components of the NO/cGMP pathway including NO, specific activators of sGC and cGKI-specific cGMP analogs all consistently inhibited the formation of Rap1-GTP. Furthermore, NO/cGMP effects were independent of the platelet agonists used for Rap1 activation. Rap1 is an important switch controlling the activity of the platelet fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$), which is required for platelet aggregation (9, 11, 16, 17). Thus regulation of Rap1 might contribute to the potent anti-aggregatory actions of NO, cGMP and cGKI. Many reports in the literature have postulated autocrine effects of platelet-derived NO on platelet aggregation (3, 35-37). In the present work Rap inhibition depended on exogenously added activators of the NO/cGMP pathway. Also, inhibition of cGMP production by ODC in the absence of NO-donors, did not reveal any basal inhibitory activity towards Rap1 (Fig. 2C) We therefore conclude that inhibition of Rap1 *in vivo* depends on endothelium-derived NO.

Our study provides evidence that cGKI is the mediator of NO/cGMP effects on Rap1. We used a number of different approaches to verify the role of cGKI including specific activators and inhibitors of cGKI as well as transfection of cGKI into cGKI-deficient cells. In addition we analyzed the phosphorylation of VASP as a measure of cGKI kinase activity. All these different approaches yielded consistent data regarding the role of cGKI. Furthermore, in all our experiments only inhibitory actions of NO/cGMP/cGKI on platelet Rap1 activation were observed. Also basal Rap1GTP levels were not elevated by NO/cGMP. cGMP and cGKI have been proposed to enhance platelet activation and aggregation under certain conditions (38, 39). However, these data were challenged by others (40, 41). The controversial data might result from unexpected side-effects of cGMP-analogs. For example, Rp-8pCPT-cGMPS was shown to inhibit thrombin-induced P-selectin expression as well as Ca^{2+} -mobilization independently of cGKI and without any effects on VASP phosphorylation (40). These cGKI-independent side-effects of Rp-8pCPT-cGMPS were evident particularly after short-term application of the cGMP-analog (1 min). In our experiments (Fig. 4) platelets were preincubated with Rp-8pCPT-cGMPS for 15 min and we never observed any inhibitory effects of Rp-8pCPT-cGMPS on ADP- or thrombin-induced Rap1-GTP formation. Furthermore, possible side-effects of Rp-8pCPT-cGMPS might not affect Rap1 activation pathways.

Conflicting results have been presented regarding the role of intracellular Ca^{2+} -release for Rap1 activation. Rap1 activation was initially considered to be Ca^{2+} - and PKC-mediated (19, 20). Later studies on ADP-induced Rap1 activation suggested either a completely Ca^{2+} -independent Rap1 activation mediated by the G_{β_i} -coupled $P2Y_{12}$ receptor and PI3K (21, 24), or a mixture of $P2Y_{12}$ - and $P2Y_1$ -mediated mechanisms (22). Our study supports the notion that both, Ca^{2+} -dependent G_{β_q} -mediated ($P2Y_1$) and G_{β_i} -mediated ($P2Y_{12}$) pathways, are involved in ADP-induced Rap1 activation in human platelets (Fig. 7). Our data imply a relative contribution of 70 % G_{β_q} -mediated versus 30 % G_{β_i} -mediated signals to the

activation of Rap1 (Fig. 5C). However, the role of G_{α_q} - and G_{α_i} -mediated signals for Rap1 activation is probably more complex. For example, AR-C69931MX, a specific $P2Y_{12}$ receptor antagonist, can inhibit the $P2Y_1$ -induced calcium response (42) suggesting that G_i -mediated pathways contribute to G_{α_q} -mediated calcium signalling. On the other hand, G_{α_q} ($P2Y_1$) probably contributes to G_{α_i} -signalling ($P2Y_{12}$) since AR-C69931MX alone is capable of blocking ADP-induced Rap1 activation completely (21). cGKI is known to inhibit Ca^{2+} -dependent platelet responses (32). However, our study as well as data by others (43) suggest that cGKI also inhibits Ca^{2+} -independent signalling.

A remaining open question is the identity of the cGKI substrate(s) mediating Rap1 inhibition. Early studies showed direct phosphorylation of Rap1 by cGKI (25, 26). However, Rap1 phosphorylation did not change its GTP-binding or GTPase activity (25). Furthermore, the slow kinetics of Rap1 phosphorylation do not correlate with the fast inhibition of platelet activation by NO/cGMP (44). The cGKI substrate VASP has been shown to be involved in platelet adhesion (45), however, NO/cGMP effects on platelet aggregation were only marginally affected in VASP-deficient mice (46, 47). Very recently, RIAM was identified, a new cytoskeletal protein capable of binding both Rap1 and VASP (48). However, in this paper no effect of VASP on Rap1 activity was described and RIAM expression in platelets was not investigated. Other known substrates of platelet cGKI include PDE5, which mediates negative feedback regulation of cGMP levels (49) and LASP and HSP27, two actin-binding proteins without any known integrin regulating functions (50, 51). In our study NO/cGMP inhibited Rap1 activation induced by many different stimuli. We conclude that the most likely target of NO/cGMP/cGKI should be a molecule common to these different signalling pathways. Possible candidates are the guanine-nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs) required for Rap1 function (Fig. 7). Many specific GEFs and GAPs for Rap1 have been identified (9), however, until now only CalDAG-GEFI/RasGRP2 expression has been demonstrated in mouse platelets (18).

Regulation of Rap1 activity is an important new aspect of NO/cGMP function because of the intimate relationship between Rap1, integrin activity and platelet aggregation. Further analysis of the mechanisms involved might lead to the development of new strategies for antithrombotic therapy.

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Figure Legends

Figure 1. NO-donors inhibit agonist-induced Rap1 activation in human platelets.

Freshly isolated washed human platelets were preincubated without or with increasing concentrations of either DEA-NO for 2 min (panel A) or DETA-NO for 1 min at 37°C (panels B and C). Then thrombin (1 U/ml, panel A), ADP (10 μ M, panel B) or collagen (50 μ g/ml) were added for 1 min. Cells were lysed and pull-down assays of Rap1 using an activation specific probe were performed. Levels of Rap1-GTP and total Rap1 as loading control were determined by Western blot. Shown are representative results of three independent experiments.

Figure 2. The NO-sensitive guanylyl cyclase is involved in Rap1 inhibition.

A, B: Washed platelets were treated without or with 10-30 μ M YC-1 for 5 or 10 min (panels A, B, respectively) at 37°C followed by addition of 1 U/ml thrombin (panel A) or 10 μ M ADP (panel B) for 1 min. Cells were lysed and Rap1-GTP was analyzed by pull-down assay. Total Rap1 as well as the phosphorylation state of VASP were ascertained by Western blotting of cell lysates.

C: Platelets were preincubated with 30 μ M ODQ for 30 min at 37°C. Then 50 μ M DETA-NO was added for 1 min followed by 50 μ g/ml of collagen I for 1 min. Rap1-GTP, total Rap1 and phosphorylated VASP were analyzed as described.

Shown are representative results of three independent experiments.

Figure 3. cGMP effects are mediated by cGKI.

Washed human platelets were incubated for 10 min with increasing concentrations of 8-pCPT-cGMP at 37°C followed by incubation with either 1 U/ml thrombin (panel A), 10 μ M ADP (panel B) or 50 μ g/ml collagen I (panel C) for 1 min. Cells were harvested and analyzed as described in the legend to Figure 2. In panel C the ADP control represents platelets

stimulated with 10 μ M ADP for 1 min. Experiments were performed at least three times with similar results.

Figure 4. NO inhibits Rap1 via cGKI.

Washed platelets were treated with 200 μ M Rp-8pCPT-cGMPS for 15 min, then 1 μ M DEA-NO was added for 1 min followed by 10 μ M ADP for various time-points. Rap1-GTP and total Rap1 were analyzed as described. Blots from three independent pull-down experiments were scanned and quantified. Shown are means \pm SEM. The amount of Rap1-GTP generated by incubation with ADP alone for 15 sec. was set to 100 %.

Figure 5. P2Y₁-independent Rap1 activation is blocked by cGMP-analogs.

ADP-induced formation of Rap1-GTP was analyzed in washed human platelets pretreated without or with rising concentrations of MRS2179, a selective P2Y₁ receptor blocker for 1 min (panel A). Furthermore, platelets were pretreated without or with various concentrations of 8-pCPT-cGMP for 10 min followed by 50 μ M MRS2179 for 1 min as indicated (panel B). Then 10 μ M ADP was applied for 1 min, cells were lysed and pull-down assays were performed with the activation specific probe for Rap1 as well as control Western blots for total Rap1 and phosphorylated VASP. Blots from three independent pull-down experiments were scanned and quantified (panel C). Shown are means \pm SEM. The amount of Rap1-GTP generated by incubation with ADP alone was set to 100 %.

Figure 6. cGKI inhibits turbulence-induced Rap1 activation in megakaryocytes.

Human MEG-01 megakaryocytes were infected with adenoviral vectors for either wildtype or catalytically inactive cGKI \square . Two days after infection cells were subjected to mixing-induced turbulence followed by 2 min rest. Then cells were lysed and levels of Rap1-GTP were determined by pull-down assay, cGKI expression as well as the phosphorylation state of

VASP were analyzed by Western blotting of cell lysates. Shown are representative results of three independent experiments.

Figure 7. Model of NO/cGMP and Rap1 signalling in platelets

Many different platelet agonists including ADP, thrombin and collagen induce the activation of Rap1. Rap1 activation involves G_q- and G_i-mediated and other pathways, which converge on specific guanine-nucleotide-exchange factors (Rap1-GEFs). GEFs induce the formation of active Rap1-GTP. Rap1 activates integrin $\alpha_{IIb}\beta_3$ required for fibrinogen binding and aggregation. NO inactivates Rap1 via soluble guanylyl cyclase (sGC) and cGMP-dependent protein kinase (cGKI). cGKI impacts on Ca²⁺-signalling (solid line) and possibly on Rap1GEFs or GTPase activating proteins of Rap1 (Rap1GAPs, dashed lines).

Figure 1

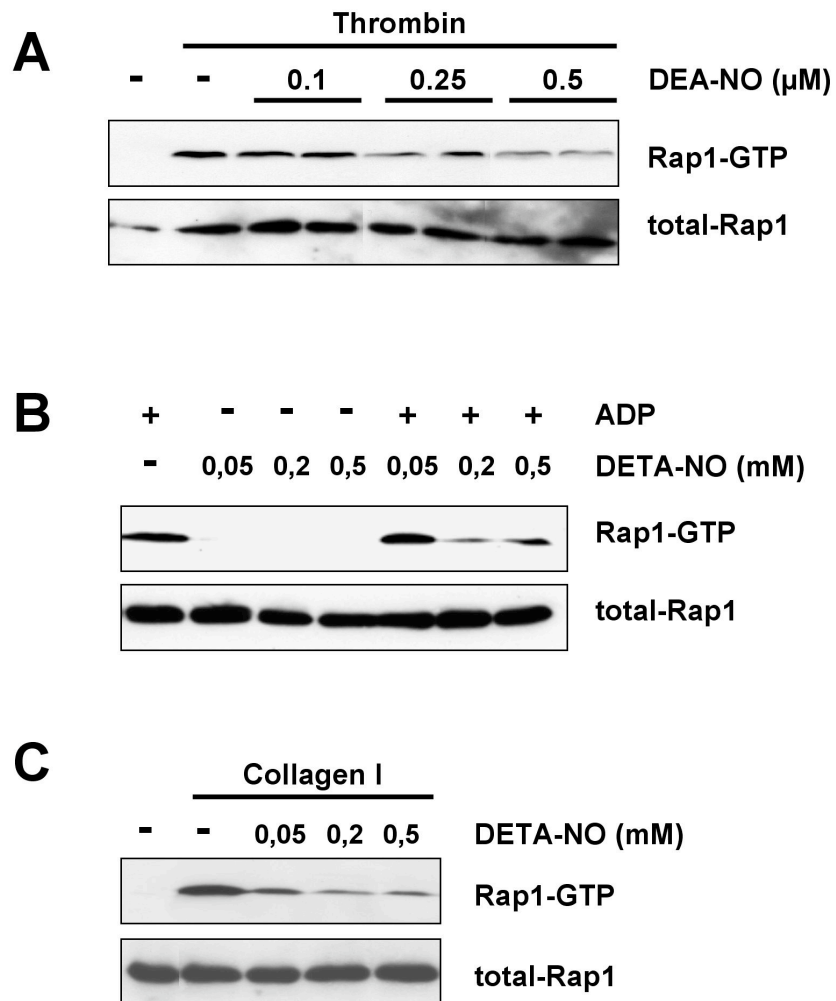


Figure 2

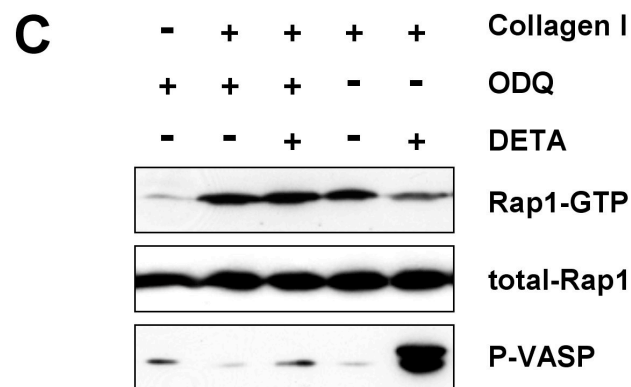
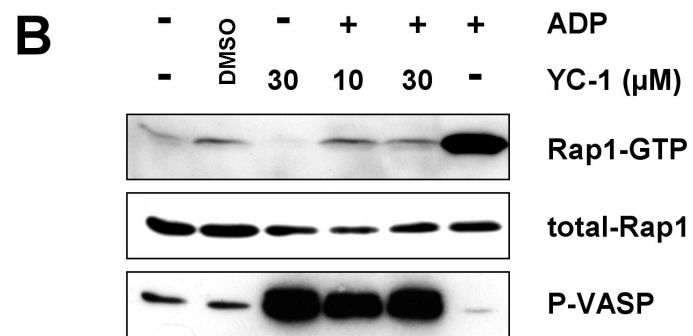
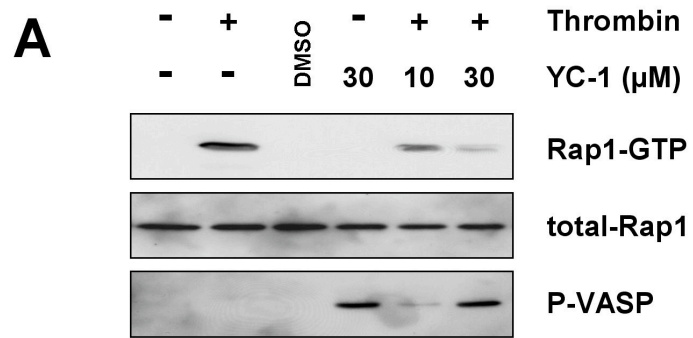


Figure 3

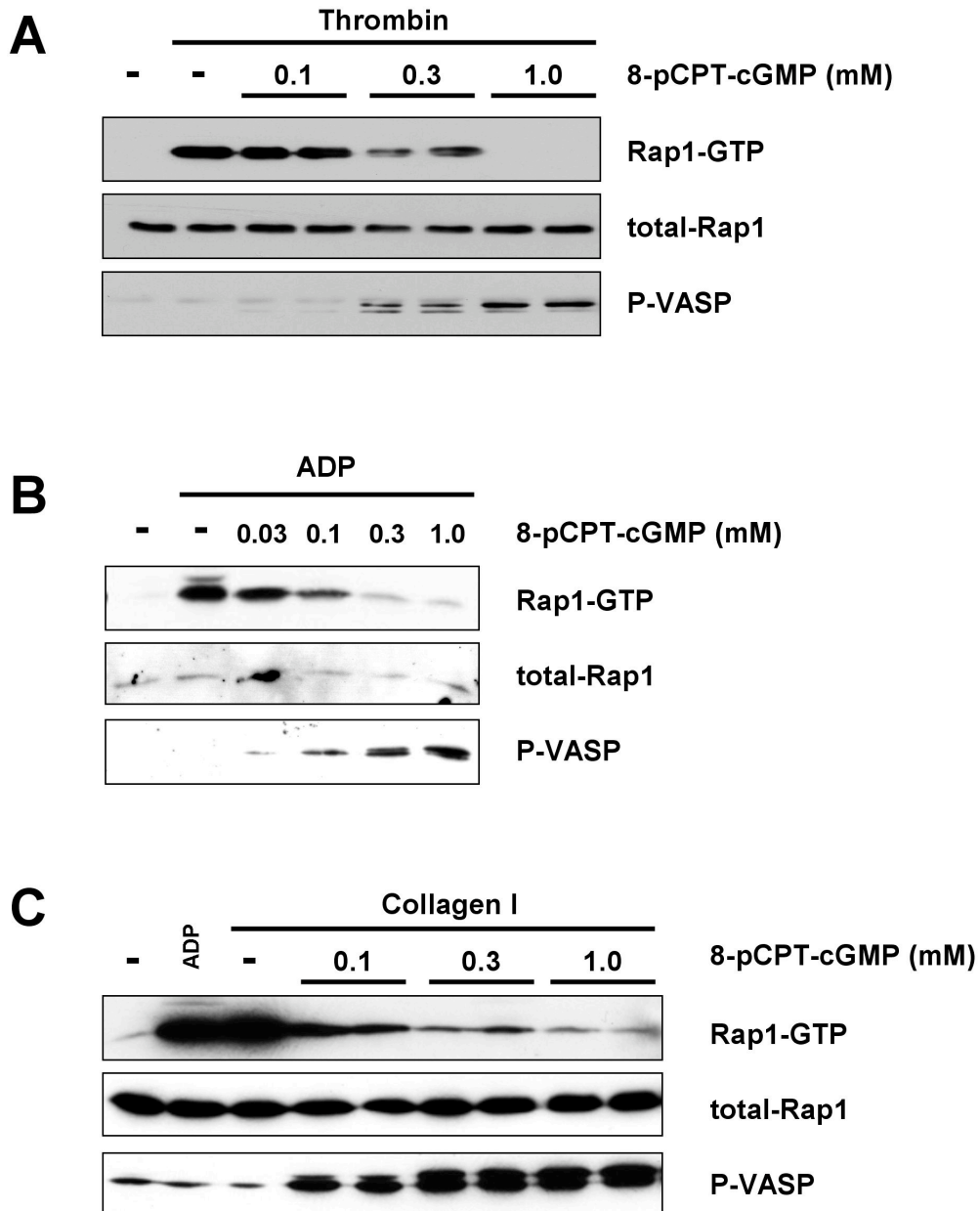


Figure 4

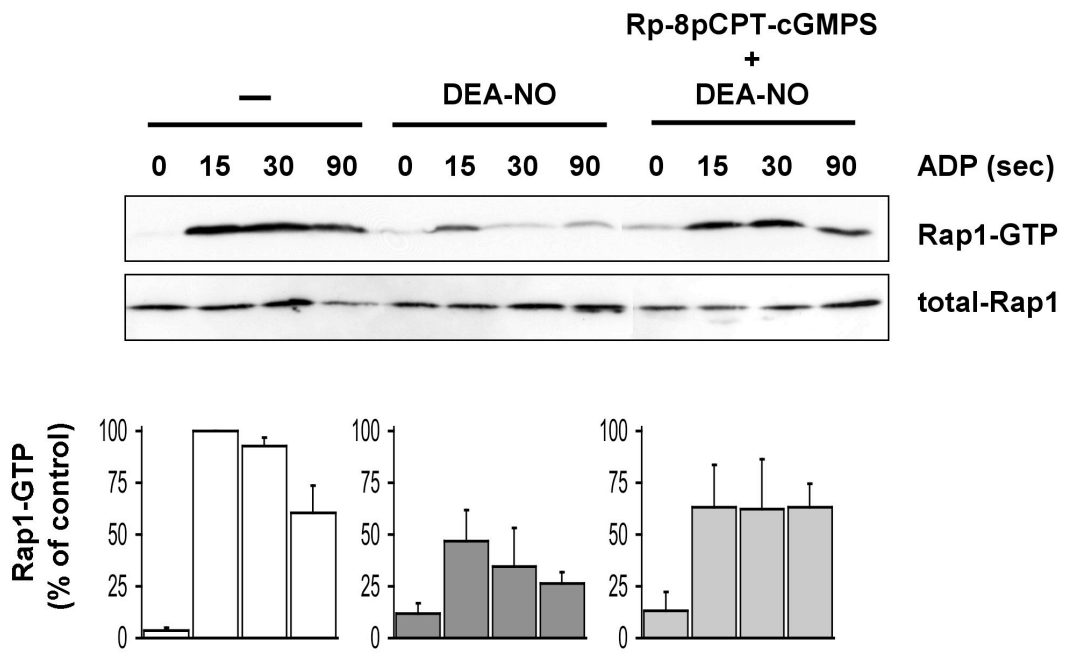


Figure 5

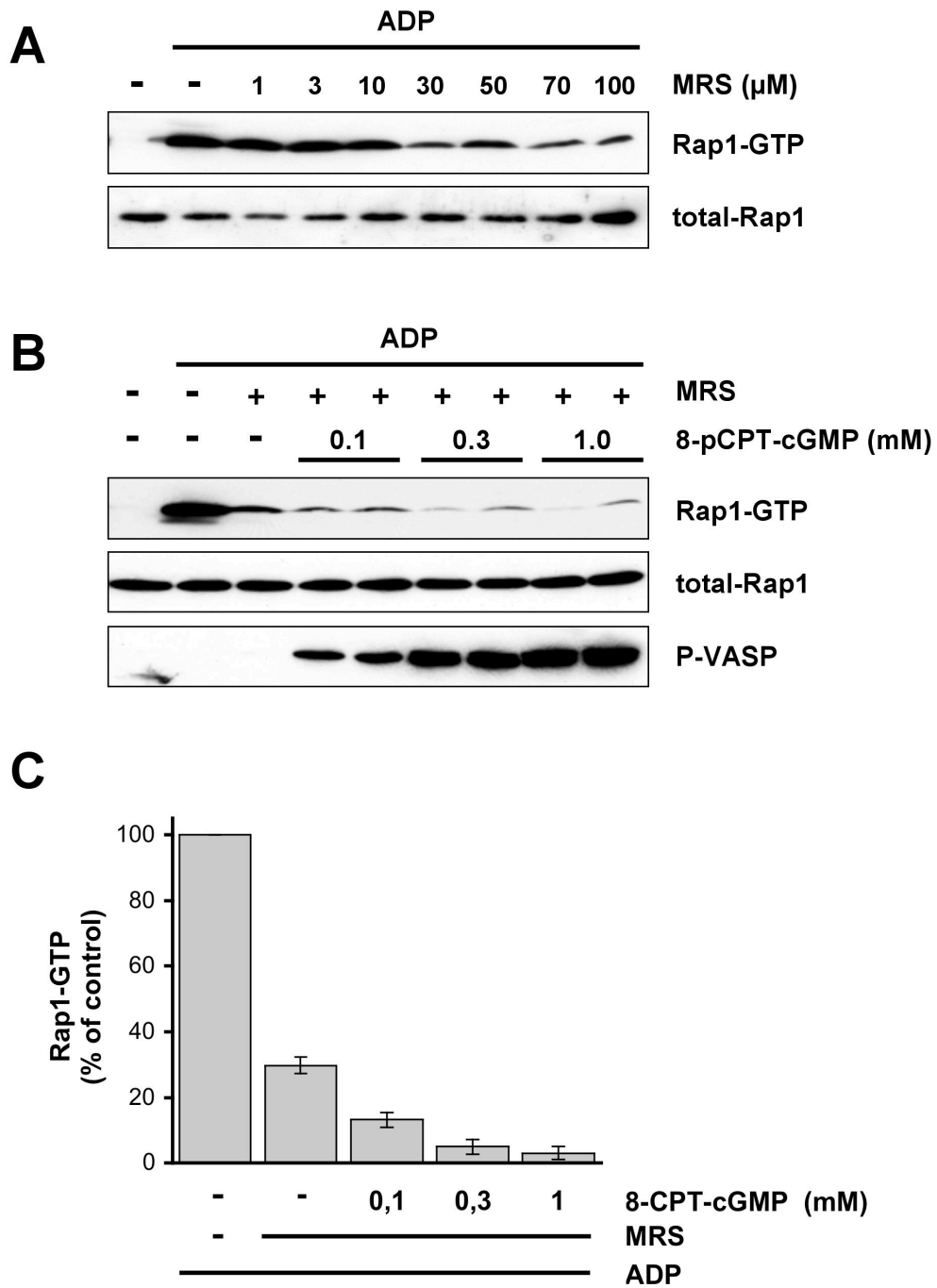


Figure 6

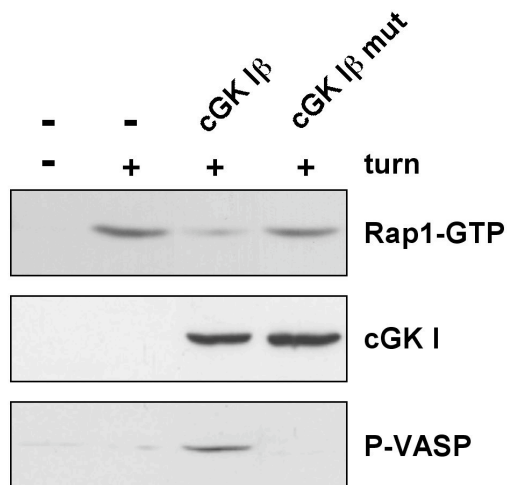


Figure 7

