IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation

and thrombus formation

Melanie Antl1, Marie-Luise v. Brühl2, Christina Eiglsperger1, Matthias Werner1,3, Ildiko Konrad2, Thomas Köcher4, Matthias Wilm5, Franz Hofmann1, Steffen Massberg2,6, and Jens Schlossmann1§

1 Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Straße 29, 80802 München, Germany
2 Deutsches Herzzentrum und 1. Medizinische Klinik, Klinikum rechts der Isar, Technische Universität München, Lazarettstraße 36, D-80636 München, Germany
3 Present address: Department of Pharmacology, College of Medicine, University of Vermont, Burlington, VT 05405, USA
4 Biological & Medical Mass Spectrometry; Uppsala Biomedical Centrum; Husargatan 3 Box 583; SE - 75 123 Uppsala, Sweden
5 Protein and Peptide Group, EMBL, Meyerhofstraße 1, 69117 Heidelberg, Germany
6 CBR Institute and Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

§ Corresponding author:
Jens Schlossmann, Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Straße 29, 80802 München, Germany
Tel.: 0049-89-4140 3265; Fax: 0049-89-4140 3261
e-Mail: Schlossmann@ipt.med.tu-muenchen.de

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Abbreviations:
IRAG, inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate; cGKI, cGMP-dependent protein kinase I; InsP3RI, inositol-1,4,5-trisphosphate receptor type I; VASP, vasodilator-stimulated phosphoprotein; NO, nitric oxide; cGMP, cyclic GMP; PGI2, prostacyclin

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ABSTRACT

Defective regulation of platelet activation/aggregation is a predominant cause for arterial thrombosis, the major complication of atherosclerosis triggering myocardial infarction and stroke. A central regulatory pathway conveying inhibition of platelet activation/aggregation is nitric oxide (NO)/cyclic GMP (cGMP) signaling via cGMP-dependent protein kinase I (cGKI). However, the regulatory cascade downstream of cGKI mediating platelet inhibition is still unclear. Here, we show that the inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) is abundantly expressed in platelets and assembled in a macrocomplex together with cGKIβ and the inositol-1,4,5-trisphosphate receptor type I (InsP3RI). cGKI phosphorylates IRAG at Ser664 and Ser677 in intact platelets. Targeted deletion of the IRAG-InsP3RI interaction in IRAGΔ12/Δ12 mutant mice leads to a loss of NO/cGMP-dependent inhibition of fibrinogen-receptor activation and platelet aggregation. Intracellular calcium transients were not affected by DEA/NO or cGMP in mutant platelets. Furthermore, intravital microscopy shows that NO fails to prevent arterial thrombosis of the injured carotid artery in IRAGΔ12/Δ12 mutants. These findings reveal that interaction between IRAG and InsP3RI has a central role in NO/cGMP-dependent inhibition of platelet aggregation and in vivo thrombosis.

Abstract: 176 Words
INTRODUCTION

Platelet activation and aggregation at foci of vascular injury is essential for primary hemostasis, but also initiates arterial thrombosis, the leading cause of myocardial infarction and stroke.\textsuperscript{1} The gaseous molecule nitric oxide (NO) is an endogenous platelet antagonist and inhibits platelet activation and aggregate formation both \textit{in vitro} and \textit{in vivo}.\textsuperscript{2,3,5} NO activates soluble guanylyl cyclases initiating a subsequent rise in platelet cyclic GMP (cGMP).\textsuperscript{2,3} Several mechanisms have been proposed by which NO/cGMP signaling abolishes platelet activation and aggregation including inhibition of G-protein coupled receptors and rearrangement of the cytoskeleton.\textsuperscript{3,6} In addition, NO/cGMP prevents inositol-1,4,5-trisphosphate (Ins\textsubscript{P\textsubscript{3}})-mediated intracellular calcium release\textsuperscript{3,6}, the critical step in the signal transduction pathway that leads to full platelet activation.\textsuperscript{7} The cGMP-dependent protein kinase type I (cGKI) is strictly required for inhibition of platelet activation by NO/cGMP.\textsuperscript{2,8} Although cGKI has been reported to inhibit intracellular Ca\textsuperscript{2+} release in platelets\textsuperscript{9-11}, the exact molecular targets downstream of cGKI involved in NO/cGMP-dependent inhibition of platelet activation have not been defined.

In smooth muscle cells, we have identified the cGKI-substrate IRAG (inositol-1,4,5-trisphosphate (Ins\textsubscript{P\textsubscript{3}}) receptor-associated cGKI substrate protein), a 125 kD protein which co-purifies in a macrocomplex together with cGKI and the Ins\textsubscript{P\textsubscript{3}} receptor (Ins\textsubscript{P\textsubscript{3}}R) type I.\textsuperscript{12} IRAG is essential for NO/cGMP-dependent smooth muscle cell relaxation, as it negatively regulates Ins\textsubscript{P\textsubscript{3}}-induced calcium release.\textsuperscript{12,13} Because of the importance of cGKI signaling in platelets, we studied here the expression and cGKI-dependent phosphorylation of IRAG in platelets and the physiological relevance of the IRAG-Ins\textsubscript{P\textsubscript{3}}RI interaction for the regulation of platelet function. We provide first evidence that the IRAG-Ins\textsubscript{P\textsubscript{3}}RI interaction mediates NO/cGMP-dependent inhibition of thrombin-induced increases in [Ca\textsuperscript{2+}], in platelets and is
the major determinant of NO/cGMP-dependent prevention of platelet aggregation *in vitro* and arterial thrombosis *in vivo*. 
MATERIAL AND METHODS

Materials. 8-pCPT-cGMP, Rp-8-Br-PET-cGMPS, Sp-5,6-DCI-cBIMPS (cBIMPS), 8-AET-cGMP-agarose, ethanolamine-agarose (Biolog), forskolin (Calbiochem), prostacyclin (Sigma), iloprost (Axxora), DEA/NO (Axxora), NO-spermine (Axxora), GEA-NO 3162 (Axxora), sodium nitroprusside (Sigma), protein A-sepharose (Sigma), \(^{33}\)P-H\(_3\)PO\(_4\) (ICN), [\(^\gamma\)-\(^{32}\)P]ATP (Amersham), secondary antibodies (Dianova), pSer239-VASP antibody, InsP\(_3\)RI antibody (Axxora). Standard chemicals were purchased from Sigma.

Preparation of human platelets. Blood from healthy volunteers was collected in ACD-buffer and then centrifuged twice (20 min, 300 g, RT). The obtained platelet rich plasma was centrifuged (15 min, 1,500 g) and the resulting platelet pellet was resuspended in HEPES-buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, 1 mM EDTA). Human platelet membranes and cytosol were isolated as described and stored at −80°C.\(^{14}\) Approval was obtained from the ethics committee of the faculty of medicine of the technical university Munich for these studies. Informed consent was provided according to declaration of Helsinki.

Phosphorylation in intact platelets. Human platelets were either incubated with HEPES-buffer alone or HEPES-buffer containing either \(^{33}\)P-H\(_3\)PO\(_4\) (1 mCi/mg platelets) or 10 mM sodium phosphate and 2 mM MgCl\(_2\) (1.5 h, 37°C) and then stimulated with diverse agents as indicated. Platelets were lysed with SDS-buffer (50 mM Tris/HCl, pH 8.0, 17.3 mM SDS, 1 mM DTT) (5 min, 95°C). After centrifugation (30 min, 20,000 g, 4°C) the supernatant was diluted in buffer (final concentration: 10 mM Tris/HCl, pH 7.2, 1.7 mM SDS, 24.1 mM sodium deoxycholate, 150 mM NaCl, 1.6 mM EDTA, 0.2 mM DTT, 10 mM sodium phosphate, 1% Nonidet P-40) including phosphatase inhibitors (50 mM NaF; 0.12 mM okadaic acid, 0.2 mM sodium vanadate) and protease inhibitors (0.3 mM PMSF, 1 mM benzamidine, 0.42 nM leupeptin) and then incubated with IRAG-specific antibodies bound to
protein A-sepharose beads (2 h, 4°C). Proteins were eluted with Laemmli-buffer, analyzed by SDS-PAGE and Western blot followed by autoradiography and/or immunodecoration with IRAG- or pSer677-IRAG-specific antibodies. For detection of Ser664-IRAG phosphorylation platelets were stimulated, lysed and then analyzed by immunoblotting with pSer664-specific antibody. Mass spectrometric analysis of phosphorylated IRAG, generation of pSer664-IRAG and pSer677-IRAG-specific antibodies and phosphorylation of IRAG mutants in COS-7 cells is described in Supplementary Methods.

**Isolation of the cGKI-complex.** Platelets were lysed in RIPA-buffer (20 mM Tris/HCl, pH 7.4, 24.1 mM sodium deoxycholate, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100) including protease and phosphatase inhibitors (20 min, 0°C). After lysis, the cGKI-complex was isolated by cGMP-agarose and analysed as described. As negative control, the precipitation reaction was performed with ethanolamine agarose.

**Measurement of platelet aggregation.** Blood from WT or IRAG\(^{\Delta 12/\Delta 12}\)-mice\(^4\) anesthetized by diethylether inhalation was collected by cardiac puncture into 200 µL Alsever's buffer (Sigma) containing 5 mM acetyl salicylic acid and 200 U/mL refludan (Schering), mixed with 500 µl buffer B (20 mM HEPES, pH 6.2, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl\(_2\), 0.36 mM NaH\(_2\)PO\(_4\)) and centrifuged (15 min, 70 g). Platelet rich plasma was incubated with 0.3 U/mL apyrase for 5 min and centrifuged (5 min, 600 g). Platelets were resuspended in buffer B (pH 7.4) at 1.5 x 10\(^5\) platelets/µl containing apyrase and 5 mM glucose (1 h, RT). For thrombin-induced aggregation, blood was drawn from isoflurane anesthetized mice and the use of acetylic acid, refludan and apyrase was omitted during the isolation of platelets. Platelets from wild-type or IRAG\(^{\Delta 12/\Delta 12}\)-mice were preincubated (5 min, 37°C) and then incubated with/without 8-pCPT-cGMP (200 µM) for 10 min, DEA/NO (300 nM - 30 µM) for 1 min, SNP (5-10 µM) for 2 min, cBIMPS (30 µM) for 5 min or with prostacyclin (5 µM) for 1 min at 37°C. Aggregation was started by collagen (5-10 µg/mL) or thrombin (0.1 U/mL).
and measured by an optical aggregometer (Chronolog) using Aggro/Link Software 5.1 (aggregation: maximal slope) and a pen recorder (shape change: area under the curve). All procedures performed on mice were approved by the German legislation on protection of animals.

**Measurement of intracellular calcium.** Platelets were isolated from isoflurane anesthetized mice, loaded at $1 \times 10^8$ platelets/mL with Fura-2 AM (1 µM) for 45 min, washed, incubated with DEA/NO (10 µM in NaOH) or Vehicle (100 µM NaOH) for 1-5 min or with 8-pCPT-cGMP (100-200 µM) for 10-30 min and then treated with mouse thrombin (0.4 U/mL). The ratio of the emission at 510 nm upon excitation at 340 nm and 380 nm wavelength (Ratio F340/380) was measured as an indicator of $[Ca^{2+}]_i$, using the Fluostar Optima Fluorometer (BMG Labtech).

**Platelet preparation for intravital microscopy.**

Donor mice of either genotype were anesthetized by inhalation of isoflurane and 850µL whole blood was collected by cardiac puncture into syringes containing 150 µL citrate buffer. Thereafter, 1 mL Tyrode’s buffer (10 mM HEPES, 1.4 M NaCl, 26 mM KCl, 121 mM NaHCO₃, 0.1% BSA, 0.1% Glucose, pH 6.5) was added and the sample was centrifuged for 20 min at 92 g. The platelet rich plasma was further incubated with 5-carboxy-fluorescein diacetate succinimidyl ester (DCF, 10 µg/mL) for 3 min, followed by centrifugation for 10 min at 1277 g. Labeled platelets were then resuspended in Tyrode’s buffer (pH 7.4) and adjusted to a final concentration of $1.5 \times 10^5$ platelets in 250 µL.

**Intravital imaging of platelet aggregation during arterial thrombosis.**

For fluorescence microscopy WT or IRAG mutant mice were anesthetized by intraperitoneal injection of a solution of midazolam (5 mg/kg body weight, Ratiopharm), medetomidine (0.5 mg/kg body weight, Pfizer) and fentanyl (0.05 mg/kg body weight, CuraMED Pharma GmbH). A polyethylene catheter was implanted into the right jugular vein for infusion of
drugs and labeled platelets, respectively, and the left common carotid artery was gently exposed. Subsequently, all animals received an intravenous bolus of NO-spermine (50 µM in NaOH), Iloprost (50 µM), or Vehicle (50 µM NaOH), as indicated. Fluorescent donor platelets of either genotype were preincubated for 2 min with either NO-spermine (50 µM in NaOH), Iloprost (50 µM), or Vehicle (50 µM NaOH) and subsequently infused iv. Thereafter, endothelial disruption of the carotid artery was initiated by ligation of the common carotid artery near the carotid bifurcation for 5 min as described in detail elsewhere.15

To directly visualize in vivo platelet aggregation in the injured carotid artery we used an intravital high-speed widefield Olympus BX51WI fluorescence microscope with a long-distance condenser and a 20x (NA 0.95) water-immersion objective. The system is equipped with Olympus MT 20 monochromator for excitation and an F-View CCD camera (Olympus). Platelet aggregation was determined using the Cap image 7.1 analysis program (Dr. Zeintl, Heidelberg, Germany) as previously described15 and is given in µm² thrombus area.

**Alexa**<sup>TM</sup>488-Fibrinogen binding. Platelets were isolated from mice anaesthetized by isoflurane inhalation, incubated at 2 x 10<sup>6</sup> platelets/mL with DEA/NO (100 nM) or iloprost (10 µM) for 2 min, 8-pCPT-cGMP (200 µM) or cBIMPS (30 µM) for 10 min at RT and then treated with mouse thrombin (0.1 U/mL) or PBS and Alexa<sup>TM</sup>488 labeled fibrinogen (12.5 µg/mL) for 10 min at RT. The samples were fixed with 1% para-formaldehyd. Fluorescence was measured using a FACScalibur flow cytometer (BD Biosciences) (excitation: 488 nm, emission: 520 nm).

**Regulation of GPIIb-IIIa activation.** Platelets were isolated from isoflurane anesthetized WT or IRAG<sup>Δ12/Δ12</sup>-mice, incubated at 5 x 10<sup>7</sup> platelets/mL with DEA-NO (100 µM in NaOH) or Vehicle (100 µM NaOH) for 2 min or with 8-pCPT-cGMP (200 µM) for 10 min at RT and then treated with mouse thrombin (0.05 U/mL) or PBS for 10 min at RT. Expression
of the activation-dependent high affinity conformation of GPIIb-IIIa (αIIbβ3 integrin) was
determined using PE-labeled JON/A antibody (Emfret, Würzburg, Germany).16,17
Fluorescence was measured on a FACScalibur flow cytometer (BD Biosciences). Data are
given as absolute increase in mean fluorescence intensity compared to resting platelets.

Calculation and statistics. All data are expressed as mean ± SEM. For the calculation of
statistical differences between two means the unpaired Student’s t-test was used. The
significance of p-value was indicated by asterisks (*, p<0.05; **, p<0.01; ***, p<0.001, ns:
not statistically significant). n indicates the number of experiments.
RESULTS

Assembly of the cGKI-macrocomplex in platelets

IRAG is expressed abundantly in the membrane fraction of platelets together with cGKIβ (Figure 1A). In human platelets only the β-isof orm of cGKI is present (Figure 1A), whereas murine platelets contain in addition a small amount of cGKIα (Supplementary Figure 1A). In both human and murine platelets, IRAG co-purifies with InsP3RI and cGKIβ on a cGMP agarose column (Figure 1B; Supplementary Figure 1C), whereas these proteins were not precipitated by the negative control column ethanolamine agarose (data not shown), indicating that platelet IRAG is assembled in a stable macrocomplex consisting of InsP3RI, IRAG and cGKIβ. The vasodilator-stimulated phosphoprotein (VASP), a cGKI substrate associated with the cytoskeleton, was not found in the purified complex suggesting that VASP is not stably associated with cGKIβ (Figure 1B).

Phosphorylation of IRAG by cGKI

We show here that platelet IRAG is phosphorylated in an NO/cGMP-dependent manner in vitro and in intact platelets. Activated cGKI phosphorylated IRAG, InsP3RI and cGKIβ in the isolated complex from platelets (Figure 1B). IRAG was also phosphorylated by cGKI when intact human platelets were preincubated with 8-pCPT-cGMP (Figure 1C) or the NO donor GEA-NO (Figure 1D). Within 1-5 min after addition of 8-pCPT-cGMP or GEA-NO, IRAG phosphorylation increased up to 2.5-fold (Figure 1C, D and data not shown). The kinetics of IRAG phosphorylation were similar to that of VASP.18
Previously, we have identified several in vitro phosphorylation sites of IRAG isolated from bovine tracheal smooth muscle membranes. To define which of these sites was phosphorylated in platelets in response to NO/cGMP, IRAG (approx. 2 µg) was purified from 8-pCPT-cGMP-treated or non-treated human platelets and analyzed by mass spectrometry using nanoelectrospray-based ion scanning (data not shown). Three phosphorylated serine residues were identified, Ser374, Ser664 and Ser677. While Ser664 was phosphorylated only in response to cGMP, Ser374 showed constitutive phosphorylation in both non-treated and treated platelets. In contrast, phosphorylation of Ser677 was observed in resting platelets (1 peptide peak) with a substantial increase in the presence of cGMP (3 peptide peaks).

We then determined the phosphorylation kinetics of the individual IRAG phosphorylation sites. We generated antibodies that specifically detect pSer664 or pSer677 phosphorylation using chemically synthesized phosphorylated peptides as immunogens (Supplementary Figure 2). These antibodies showed that both cGMP and NO strongly increase the phosphorylation at Ser664 and Ser677 in human platelets (Figure 2A, B; Supplementary Figure 2C). The cGKI inhibitor Rp-8-Br-PET-cGMPS prevented NO/cGMP-induced phosphorylation (Figure 2A, B and data not shown) of Ser664 and Ser677 suggesting that both serines were in fact phosphorylated by cGKI. The specificity of Rp-8-Br-PET-cGMPS on NO signaling was demonstrated previously as Rp-8-Br-PET-cGMPS inhibited NO/cGMP-induced VASP phosphorylation and reversed NO-mediated inhibition of platelet aggregation. Consistently, we detected that Rp-8-Br-PET-cGMPS (200 µM, 20 min preincubation) suppressed the effect of DEA/NO (300 nM, 1 min preincubation) on collagen (10 µg/mL)-induced platelet aggregation in human platelet rich plasma, whereas Rp-8-Br-PET-cGMPS alone did not significantly affect platelet aggregation. [Aggregation (% of control): +NO: 35.1±5.8 (n=7); +NO/Rp: 78.2±2.8 (n=7); Rp: 90.7±1.7 (n=4)].
Phosphorylation of Ser664 and Ser677 occurred rapidly reaching a maximum within 1-3 min after the addition of NO (Figure 2C and data not shown). Likewise, the stable cGMP analogue 8-pCPT-cGMP lead to a strong and long lasting phosphorylation at Ser664. The time course of Ser664 and Ser677 phosphorylation matched that of NO/cGMP-stimulated $^{33}$P-phosphorylation of IRAG (Figure 1C, D) and paralleled the phosphorylation of VASP at Ser239 (Figure 2). These results indicate that IRAG is expressed in platelets and is phosphorylated by cGKI at Ser664 and Ser677 in intact platelets. Ser677 of human IRAG is homologous to the bovine Ser696.12 Phosphorylation of IRAG at the Ser677 homologue is essential for cGKI-induced inhibition of InsP$_3$-stimulated Ca$^{2+}$ release$^{13}$, suggesting that IRAG phosphorylation mediates NO/cGMP-dependent inhibition of agonist-induced Ca$^{2+}$ release in platelets.$^{9-11,21}$
Role of IRAG in NO/cGMP-dependent platelet signaling

Next, we investigated the functional relevance of IRAG for NO/cGMP-dependent signaling in platelets. We used murine IRAG$^{\Delta 12/\Delta 12}$ loss-of-function mutants which have a targeted deletion of the N-terminal part of the coiled-coil domain of IRAG required for the interaction with the InsP$_3$RI$^4$. IRAG$^{\Delta 12}$ protein expression was reduced by approximately 80% in homozygous IRAG$^{\Delta 12/\Delta 12}$ platelets, whereas the expression of cGKI$\beta$ and cGKI$\alpha$ was not significantly affected (Supplementary Figure 1A). Furthermore, NO and cGMP-dependent phosphorylation of VASP-Ser239, was identical in WT- and mutant platelets suggesting the same cGKI activity in both types of platelets$^{22,23}$ (Supplementary Figure 1B). In IRAG$^{\Delta 12/\Delta 12}$ platelets, the InsP$_3$RI did not co-purify with IRAG and cGKI (Supplementary Figure 1C) indicating that the InsP$_3$RI is not integrated into the IRAG$^{\Delta 12}$-cGKI-macrocomplex in mutant platelets as previously reported for smooth muscle.$^4$

Both, WT and IRAG$^{\Delta 12/\Delta 12}$ platelets - which were isolated in the presence of acetyl salicylic acid, the thrombin inhibitor refludan and the ATP/ADP degrading enzyme apyrase to prevent prior platelet activation - readily aggregated in response to collagen (Figure 3A). Pretreatment of WT platelets with 8-pCPT-cGMP, DEA/NO and SNP virtually abolished aggregation (Figure 3A, B). Aggregation of mutant IRAG$^{\Delta 12/\Delta 12}$ platelets was not affected by 8-pCPT-cGMP and weakly inhibited at low or high concentrations of the two different NO donors DEA/NO (300 nM, 30 µM) or SNP (5 µM, 10 µM). However, aggregation of wild type and mutant platelets was strongly inhibited by the cAMP analogue cBIMPS or prostacyclin (Figure 3A, B; Supplementary Figure 3). Different concentrations of the agonist collagen (5 and 10 µg/mL) were used to exclude the possibility that an altered agonist response of the mutant was responsible for the abolished NO/cGMP dependent inhibition of platelet aggregation. Furthermore, when cross-activation pathways were not blocked omitting...
acetyl salicylic acid, refludan and apyrase during platelet isolation, NO- and cGMP-mediated inhibition of collagen-induced platelet aggregation was still strongly suppressed in IRAG homosexuals allele platelets in comparison to WT platelets (% of control: WT+cGMP (n=4) 49.3±2.3, WT+SNP (n=6) 46.6±3.7, Δ12+cGMP (n=10) 72.4±4.6, Δ12+SNP (n=9) 67.7±2.8; cGMP: 200 µM 8-pCPT-cGMP, SNP: 5 µM SNP). In contrast to aggregation, collagen-induced shape change was only affected by cAMP (Supplementary Figure 3), but not by NO/cGMP/IRAG (data not shown).

Furthermore, we tested whether induction by another agonist e.g. thrombin, which leads to Gq-activation and thereby stimulation of phospholipase Cβ24, can be affected by IRAG signaling. Both, NO- and cGMP-mediated inhibition of thrombin-induced platelet aggregation was suppressed by the IRAG mutation (Figure 3B). Therefore, it can be concluded that signaling via IRAG is a major pathway of NO/cGMP impeding platelet aggregation.

Previously, we have reported that apart from preventing platelet aggregation the NO/cGMP/cGKI cascade negatively regulates agonist-induced activation of the platelet fibrinogen receptor GPIIb-IIIa.8 Both, cGMP and NO significantly attenuated thrombin-induced fibrinogen binding to WT, but not to IRAG homosexuals allele platelets (Figure 3C, left panel). Correspondingly, direct fibrinogen receptor GPIlb-IIIa activation by thrombin was only slightly reduced by cGMP and NO in mutant platelets in contrast to wild type platelets (Figure 3C, right panel). Contrary, prostacyclin or cAMP reduced fibrinogen binding to a similar extent in both WT and IRAG homosexuals allele platelets (Figure 3C, left panel).

Furthermore, preincubation of mutant platelets with 8-pCPT-cGMP or DEA/NO hardly affected thrombin-induced calcium transients in contrast to wild type platelets (Figure 3D, E). Together these results indicated that IRAG is specifically involved in the cGMP/cGKI signaling cascade leading to inhibition of platelet activation and aggregation via...
suppression of intracellular calcium transients, while the cAMP/cAK cascade is unaffected by the IRAG mutation.
Figure 3

D

WT

Δ12/Δ12

Ratio [F340/F300] au

Ratio [F340/F300] au

0 1 2 3

0 1 2

t (min)

CTR cGMP

CTR cGMP

CTR NO

CTR NO

Ratio [F340/F300] au

Ratio [F340/F300] au

0 1 2

0 1 2

t (min)

E

WT

Δ12/Δ12

ΔRatio (% of control)

ΔRatio (% of control)

100 80 60 40 20 0

100 80 60 40 20 0

200 μM, 10 min 100 μM, 20 min 100 μM, 30 min

1 min 5 min

8-pCPT-cGMP DEA/NO

* **
IRAG function in NO/cGMP-dependent prevention of thrombus formation

To further dissect the biological role of platelet IRAG, we directly visualized platelet aggregation and thrombus formation following vascular injury using in situ high-speed intravital microscopy of the mouse carotid artery. In the injured carotid artery, WT and IRAG$^{\Delta 12/\Delta 12}$ platelets readily adhered (data not shown) and aggregated to a similar extent at the site of injury leading to local thrombus formation (Figure 4A, B). The stable NO analogue NO-spermine abolished platelet aggregate formation in WT mice. In striking contrast, NO did not affect thrombus formation in IRAG$^{\Delta 12/\Delta 12}$ mutants (Figure 4B; Supplementary movies S4-S7). Infusion of donor platelets into heteroacceptor mice (IRAG$^{\Delta 12/\Delta 12}$ platelets in WT mice, WT platelets in IRAG$^{\Delta 12/\Delta 12}$ mice) revealed that a defective function of the mutant platelets was responsible for the lack of NO-mediated suppression of arterial thrombus formation (Figure 4C). These results confirm that platelet IRAG is essential for NO/cGMP-dependent inhibition of platelet activation and prevention of arterial thrombosis. The contribution of IRAG is specific for cGMP/cGKI signaling since cAK activation by iloprost prevented platelet aggregation to a similar extent in both IRAG$^{\Delta 12/\Delta 12}$ mutants and WT mice (Figure 4A, B).
Figure 4

A

WT

+ PBS

+ NO

+ PGI₂

Δ12/Δ12

+ PBS

+ NO

+ PGI₂

B

Platelet aggregation [μm²]

WT

Δ12/Δ12

PBS

Vehicle

NO

PGI₂

***

C

Platelet aggregation [μm²]

WT

Δ12/Δ12

WRs

Recipient

NO

+ +

Δ12/Δ12

WT
DISCUSSION

The results of this manuscript clearly establish IRAG as a major player in the inhibition of platelet aggregation by the NO/cGMP signaling cascade. They resolve, at least in part, the signaling cascade downstream of cGKI in platelets. As already shown for smooth muscle, IRAG is assembled in a macrocomplex in platelets consisting of IRAG, cGKI, and InsP₃RI. IRAGΔ12 mutation inhibits the interaction with the InsP₃RI. However, based on in vitro and in vivo aggregation experiments and on calcium measurements there are no indications that mutant IRAGΔ12 results in enhanced IRAG function. Furthermore, the IRAG mutation does not alter cGKIβ function per se, as cGKIβ still interacts with the mutant IRAG and is still active (see Supplemental Figure S1). However, although our experiments suggest that the functional effect of the deletion mutant results from the defective inhibition of intracellular calcium rise by NO/cGMP in platelets, unknown alternative effects of this deletion mutation cannot be totally excluded.

Other potential cGKI substrates expressed in platelets, including the Rap1-activating GTPase Rap1-GAP2, HSP27 and VASP, did not copurify with cGKI and more importantly did not compensate for the loss of IRAG-dependent signaling. The NO/cGMP-mediated inhibition of platelet aggregation ex vivo and fibrinogen receptor activation was not completely suppressed by the IRAG mutation. Therefore, other cGKI substrates might be involved in different cGKI-signaling pathways which play a minor role in the inhibition of platelet aggregation. Most likely, these additional substrates do not include VASP as suggested by results obtained with an inactivated VASP gene. NO attenuated thrombin-induced aggregation of wild type platelets by more than 70%, while thrombin-induced integrin αIIbβ3 activation was reduced by only 26%. This indicates a non-linear correlation between (1) inhibition of platelet integrin activation (as determined by flow cytometry) and (2) attenuation of platelet aggregation.

Furthermore, IRAG is obviously involved in the NO-dependent prevention of arterial thrombus formation at sites of vascular lesion but does not alter the basal platelet aggregation at the injured artery. Interestingly, inactivation of the cGKI gene increased platelet adherence to the endothelium after ischemia/reperfusion supporting the above notion that cGKI may signal through different substrates in platelets as already shown for the situation in smooth
muscle (see 5). The physiological significance of these additional pathways needs to be established.

IRAG is linked to the InsP$_3$RI in platelets through its coiled-coil domain and is phosphorylated by cGKI at Ser677. Phosphorylation of the Ser677 homologue of human IRAG is responsible for cGKI-induced inhibition of InsP$_3$-stimulated Ca$^{2+}$ release. In IRAG mutant platelets with a defective IRAG-InsP$_3$RI interaction, the NO/cGMP-mediated suppression of intracellular calcium transients was abolished. The present study, therefore, provides the first evidence that modulation of Ca$^{2+}$ level through NO/cGMP in platelets involves IRAG-InsP$_3$RI signaling. Furthermore, the results support the notion that NO acting via cGMP/cGKI prevented agonist-induced increases of platelet calcium, a crucial step in the process of platelet activation. Recently, the inhibitory function of cGMP on platelet activation was challenged by observations that cGMP might induce a biphasic response of platelets first promoting and later inhibiting platelet aggregation. The results of this and other studies have been questioned by others. It is not clear why different groups arrived at contradictory results. The result of this paper clearly identifies a possible alternative, i.e. the difference in cGMP level that led to phosphorylation of different cGKI targets such as IRAG, VASP or thromboxane receptor $1\alpha$. The lack of VASP in the human complex purified by a cGK-specific column supports the notion that phosphorylation of this target might require high concentrations of active cGKI whereas IRAG could be phosphorylated already at intermediate concentrations of active cGKI.

cGMP-independent mechanisms including nitrosylation of proteins, activation of ADP-ribosyltransferases or transactivation of cAMP-dependent protein kinase were reported to be induced by some NO donors at high concentrations. Several different NO-donors and a cGMP-analogue, which is established in platelets, were used in this study to exclude the possibility that cGMP-independent effects of the NO donor or the cGMP analogue were
responsible for the phosphorylation of IRAG and the deficient NO/cGMP-mediated inhibition of the IRAG mutant platelet function.

In our experiments, the cGK-inhibitor Rp-8-Br-PET-cGMPS does not affect platelet aggregation without exogenous cGMP or NO. However, other groups reported\textsuperscript{31,36} that Rp-8-Br-PET-cGMPS inhibited platelet aggregation in the absence of NO/cGMP elevating compounds. It might be that the divergence to our results is based on different platelet reactivity and experimental conditions. Furthermore, it cannot be excluded that Rp-8-Br-PET-cGMPS affected signalling mechanisms independent of cGMP kinase because Marshall et al. showed an effect of cGMP analogues in the absence of cGKI.\textsuperscript{31}

Importantly, we report here that IRAG is dispensable for the inhibition of aggregation through prostacyclin/cAMP which is an important cascade impeding platelet function.\textsuperscript{6} Although cAMP-dependent phosphorylation of the InsP\textsubscript{3}R has been reported\textsuperscript{9,37,38}, recent evidence suggest that cAMP-dependent phosphorylation of the InsP\textsubscript{3}RI may increase the release of Ca\textsuperscript{2+}.\textsuperscript{39} Therefore, the cascade through which cAMP kinase inhibits platelet activation and aggregation remains to be established.

In conclusion, the present manuscript shows that NO/cGMP acting via IRAG prevents arterial thrombus formation and thereby arterial thrombosis, the major cause of morbidity and mortality in industrialized countries.

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FOOTNOTE

\textsuperscript{i} Human IRAG: GenBank accession number NP_006060
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FIGURE LEGENDS

Figure 1. cGKI macrocomplex and IRAG phosphorylation in human platelets. (A) Identification of IRAG and cGKIβ in human platelets (P-M: platelet membranes, P-C: platelet cytosol) by immunoblot analysis with specific antibodies directed against IRAG, cGKIα or cGKIβ. (B) Isolation and phosphorylation of the ternary complex of IRAG, cGKI and InsP₃RI in human platelets. The complex was isolated using cGMP-agarose beads and then phosphorylated by the addition of 8-pCPT-cGMP (3 µM) and [γ-³²P]ATP. ³²P-phosphorylation was analyzed by autoradiography (AR) and immunoblot analysis (IB) was performed with specific antibodies. The positions of molecular weight markers are indicated. Note, that the cGKI substrate VASP was not assembled in the cGKI complex. (C; upper panel). ³³P-phosphorylation of IRAG in intact human platelets stimulated with the cGMP analogue 8-pCPT-cGMP (100 µM) for 1 to 30 min. (C; lower panel) Statistics of phosphorylation results. (D) Stimulation of human platelets with the nitric oxide donor GEA-NO (100 µM). As control, in each experiment the phosphorylation of IRAG was compared with that of Ser239-VASP, determined by immunoblot analysis with pSer239-VASP specific antibodies. Equal amounts of total IRAG or VASP in the different lanes were checked by immunoblotting with IRAG- and VASP-specific antibodies (Figures 1, C and D).

Figure 2. Analysis of IRAG phosphorylation in intact human platelets with pSer664-IRAG and pSer677-IRAG antibodies. (A,B) Phosphorylation of IRAG stimulated in human platelets by 8-pCPT-cGMP (100 µM, 30 min) or by DEA/NO (10 µM, 1 min). Reactions were preincubated with the cGMP kinase inhibitor Rp-8-Br-PET-cGMPS (200 µM, 20 min) where indicated. IRAG phosphorylation was analyzed by pSer664-IRAG antibody (A) or
pSer677-IRAG antibody (B). Control conditions were performed for 8-pCPT-cGMP adding H₂O and for DEA/NO adding a final concentration of 1 mmol/L NaOH to the reaction. (C, upper panel) Kinetics of IRAG phosphorylation in human platelets by 8-pCPT-cGMP or by DEA/NO. IRAG phosphorylation was analyzed by pSer664-IRAG antibody. (C, lower panel) Statistics of phosphorylation results in % of maximal Ser664-phosphorylation. Phosphorylation of VASP analyzed by the phosphospecific pSer239-VASP antibody and immunodecoration with IRAG- and VASP-specific antibodies are shown for comparison.

**Figure 3. Ex vivo analysis of wild type and IRAGΔ12/Δ12 platelets.** (A) Effect of 8-pCPT-cGMP (200 µM), DEA/NO (30 µM), cBIMPS (30 µM) or prostacyclin (5 µM) on collagen-induced aggregation of wild type (WT) and IRAGΔ12/Δ12 (Δ12/Δ12) platelets. Representative traces of wild type and IRAGΔ12/Δ12 platelets are shown. The arrow indicates the addition of collagen (10 µg/mL) [CTR: control without addition of 8-pCPT-cGMP, DEA/NO, cBIMPS or prostacyclin]. (B) Statistical evaluation of 8-pCPT-cGMP (200 µM), DEA/NO (0.3 µM, 30 µM) or SNP (5 µM, 10 µM) on platelet aggregation induced by collagen (5 or 10 µg/mL, as indicated) or thrombin (0.1 U/mL). (C) Left panel: Statistical evaluation of fibrinogen binding to wild type and IRAGΔ12/Δ12 platelets after pre-treatment with 8-pCPT-cGMP (200 µM), DEA/NO (100 nM), iloprost (10 µM) or cBIMPS (30 µM) followed by induction of fibrinogen receptor activation with thrombin (0.1 U/mL). Right panel: Statistical evaluation of GPIIb-IIIa activation of wild type and IRAGΔ12/Δ12 platelets after pre-treatment with 8-pCPT-cGMP (200 µM) or DEA/NO (100 µM) followed by platelet activation with thrombin (0.05 U/mL). (D) Effect of 8-pCPT-cGMP (200 µM, 10 min preincubation) or DEA/NO (10 µM, 1 min preincubation) on thrombin-induced calcium release of Fura-2 AM (1 µM) loaded wild type and IRAGΔ12/Δ12 platelets. Representative traces of wild type and IRAGΔ12/Δ12 platelets.
are shown. The arrow indicates the addition of thrombin (0.4 U/mL) [CTR: control without addition of 8-pCPT-cGMP, DEA/NO; au: arbitrary units].

(E) Statistical evaluation of thrombin-induced calcium release in wild type and IRAG^{Δ12/Δ12} platelets. Fura-2 AM (1 µM) loaded platelets were incubated with DEA/NO (10 µM, 1-5 min) or 8-pCPT-cGMP (100-200 µM, 10-30 min) and then stimulated with thrombin (0.4 U/mL).

Figure 4. In vivo analysis of arterial thrombosis in wild type and IRAG^{Δ12/Δ12} mice. (A) Thrombus formation in the injured carotid artery of wild type (WT) and IRAG^{Δ12/Δ12} (12/Δ12) mice in the absence or presence of NO (50 µM NO-spermine) or PGI\textsubscript{2} (50 µM iloprost). (Arrowheads: thrombi; arrows: single, adherent platelets). (B) Statistical evaluation of thrombus formation as measured by the size of platelet aggregates [μm\textsuperscript{2}]. Analysis was performed with n=6-9 PBS-, Vehicle (NaOH)-, NO- or PGI\textsubscript{2}-treated WT or IRAG mutant mice. (C) Thrombus formation after infusion of IRAG^{Δ12/Δ12} or WT platelets into the injured carotid artery of WT or IRAG^{Δ12/Δ12} mice (recipient), respectively, in the presence of NO (50 µM NO-spermine) (Arrowheads: thrombi, n=4). Representative videoclips of thrombus formation in WT or IRAG^{Δ12/Δ12} mice from (A) in the presence or absence of nitric oxide are available as Supplementary movies S4-S7.
IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation

Melanie Antl, Marie-Luise v. Bruhl, Christina Eiglsperger, Matthias Werner, Ildiko Konrad, Thomas Kocher, Matthias Wilm, Franz Hofmann, Steffen Massberg and Jens Schlossmann