Increased Adhesion and Aggregation of Platelets Lacking Cyclic Guanosine 3′,5′-Monophosphate Kinase I

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Summary

Atherosclerotic vascular lesions are considered to be a major cause of ischemic diseases, including myocardial infarction and stroke. Platelet adhesion and aggregation during ischemia–reperfusion are thought to be the initial steps leading to remodeling and reocclusion of the postischemic vasculature. Nitric oxide (NO) inhibits platelet aggregation and smooth muscle proliferation. A major downstream target of NO is cyclic guanosine 3′,5′-monophosphate kinase I (cGKI). To test the intravascular significance of the NO/cGKI signaling pathway in vivo, we have studied platelet–endothelial cell and platelet–platelet interactions during ischemia/reperfusion using cGKI-deficient ($cGKI^{-/-}$) mice. Platelet cGKI but not endothelial or smooth muscle cGKI is essential to prevent intravascular adhesion and aggregation of platelets after ischemia. The defect in platelet cGKI is not compensated by the cAMP/cAMP kinase pathway supporting the essential role of cGKI in prevention of ischemia-induced platelet adhesion and aggregation.

Key words: fluorescence microscopy • endothelial cell • microcirculation • nitric oxide • cyclic guanosine 3′,5′-monophosphate–dependent protein kinase

Under physiological conditions, the endothelial cell layer acts as a nonthrombogenic surface. However, in response to pathological stimuli, both endothelial cells and platelets may become proadhesive/procoagulant, leading to platelet adhesion to the endothelium and to subsequent platelet aggregation. Nitric oxide (NO)[1] is of major importance for the homeostasis of platelet–endothelium and platelet–platelet interactions under both physiological and pathophysiological conditions (1). NO regulates vascular tone (2) and, together with prostacyclin, inhibits adhesion, activation, and aggregation of platelets to injured endothelial cells (3, 4). Although most of the NO effects on platelets are thought to be mediated by activation of a soluble guanylyl cyclase and a subsequent rise in intracellular cyclic guanosine 3′,5′-monophosphate (cGMP) (5), the exact pathway involved in mediating NO-dependent inhibition of platelet adhesion/aggregation remains controversial.

cGMP kinase I (cGKI) is one of the established downstream targets of the NO/cGMP signaling cascade. Platelets, as well as endothelial cells, express high concentrations of cGKI (6–8). In vitro cGMP acting via cGKI lowers cytosolic Ca^{2+} concentrations in platelets, inhibits platelet activation by potent agonists, and initiates phosphorylation of target proteins, including the vasodilator-stimulated phosphoprotein (VASP) and the thromboxane receptor (9–11). However, the in vivo importance of cGKI-dependent signaling for the homeostasis of platelet–endothelium interactions remains to be established, since an identical role has been assigned to the prostacyclin/cAMP/cAMP kinase (cAK) pathway (12).

Interactions between circulating platelets and the vascular wall are required for maintenance of vascular integrity and hemostasis. However, in certain pathophysiological processes, particularly ischemia/reperfusion (I/R), the adhesion and aggregation of platelets may also contribute to

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[1]*Abbreviations used in this paper:* cAK, cAMP-dependent protein kinase; cGKI, cGMP-dependent protein kinase I; cGMP, cyclic guanosine 3′,5′-monophosphate; I/R, ischemia/reperfusion; NO, nitric oxide; PRP, platelet-rich plasma; VASP, vasodilator-stimulated phosphoprotein.

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vascular injury (13–16). After I/R, platelets are recruited to the postischemic vasculature early after the onset of reperfusion (17), leading to luminal narrowing and eventually reocclusion (18–20). To evaluate the biological role of cGKI in the homeostasis of platelet–endothelial cell and platelet–platelet interactions in vivo, we have analyzed platelet adhesion and aggregation during I/R using a cGKI negative mouse line (8).

Materials and Methods

Animals. cGKI-deficient ($cGKI^{-/-}$) mice were generated as previously described (8) and bred and maintained at the animal facility of the Institut für Pharmakologie und Toxikologie (TU München). For experiments, 4–8-wk-old wild-type and $cGKI^{-/-}$ mice of either sex on 129sv background were used (litter- or age-matched animals). All experimental procedures performed on these mice were approved by the German legislation on protection of animals.

Western Blot Analysis and Immunoblotting. Western blot analyses of cGKI and cAK catalytic subunits cAKα and cAKβ in platelets of wild-type, heterozygous, and $cGKI^{-/-}$ mice were carried out as previously reported (21). To assess the effect of cAK and cGK activation on VASP phosphorylation, platelet-rich plasma (PRP) was prepared as described below and incubated for 20 min at room temperature in the presence of 0.1% DMSO (control), or the specific activators of cAK and cGK, cBIMPS (0.1 mM; Biolog Life Science) and 8-pCPT-cGMP (0.1 mM; Biolog Life Science), respectively. Platelets were then pelleted and lysed in Laemmli buffer. VASP phosphorylation was assessed by the mobility shift of phosphoVASP (9) and detected by immunoblotting with a VASP-specific antibody (Dianova).

Preparation of Platelets for the Assessment of Aggregation, Shape Change, and Serotonin Release. 4–8-wk-old mice of either sex and genotype were anesthetized by chloroform inhalation and their chests were opened. After intracardial injection of buffer A (75 μl buffer [138 mM NaCl, 1.0 mM $MgCl_2$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 20 mM Hepes, and 5 mM glucose, pH 7.4] containing heparin [300 U/ml]), blood was collected by cardiac puncture. Whole blood was diluted with buffer B (two parts buffer supplemented with 30 mg/ml BSA and 30 U/ml heparin), and centrifuged for 10 min at 100 *g*. PRP was carefully removed and replaced by an equal volume of buffer B. After mixing gently, samples were centrifuged again for 10 min at 100 *g* and PRP was removed. The resulting PRP fractions were pooled and centrifuged at 1,000 *g* for 10 min. The pellet was resuspended in buffer B to give a final density of $1–3 \times 10^5$ platelets/μl.

Measurement of Platelet Aggregation, Shape Change, and Serotonin Release In Vitro. To evaluate the role of cGKI in the regulation of platelet function in vitro (aggregation, shape change, and serotonin release), PRP was incubated for 20 min with 0.1% DMSO (control), 0.1 mM 8-pCPT-cGMP, or 0.1 mM cBIMPS. Aggregation was started by addition of collagen (5 μg/ml) and followed in an aggregometer (Chronolog). Aggregation and shape change were measured by recording the light transmission, as previously described (22). To assess platelet serotonin release, the PRP was loaded with 37 MBq/ml [^{3}H]5-hydroxytryptamine (5-HT; Amersham Healthcare) for 1 h at 37°C. Thereafter, the PRP was centrifuged for 10 min at 1,000 *g*, and the supernatant was removed. The pellet was resuspended in buffer A at a density of $1–3 \times 10^5$ platelets/μl. After 30 min at room temperature, platelets were incubated for 20 min at 37°C as indicated above and stimulated with collagen (5 μg/ml) for 5 min. After centrifugation, the amount of [^{3}H]5-HT released into the supernatant was determined by liquid scintillation counting.

Preparation of Platelets for Intravital Microscopy. To study platelet adhesion/aggregation during I/R in vivo, blood (0.8–1.0 ml) from an anesthetized donor mouse of either genotype was collected by cardiac puncture and added to a solution containing 500 μl Ca^{2+}- and Mg^{2+}-free PBS (PAN Systems), 200 μl citrate buffer (100 mM dextrose, 2.6 mM citric acid monohydrate, and 2.7 mM tri-sodium citrate dihydrate) and 15 μl prostaglandin (PG)E_1 (Sigma-Aldrich) (50 μg/ml). After addition of 100 μl fluorescent dye rhodamine 6G (Sigma-Aldrich) (0.5 mg/ml) to label platelets ex vivo, the whole blood was centrifuged for 10 min at 100 *g* and PRP was carefully isolated. PRP was added to a solution containing 1,000 μl PBS (Ca^{2+} and Mg^{2+} free), 30 μl PGE_1, and 200 μl citrate buffer. After centrifugation for 10 min at 1,000 *g*, the supernatant was removed and the resulting rhodamine-labeled pellet was resuspended in 500 μl PBS (Ca^{2+} and Mg^{2+} free). A 50-μl sample of the rhodamine-labeled platelet suspension was analyzed by using a flow cytometer (FACSort®; Becton Dickinson) and an A^C T counter (Coulter Corp.) to determine the purity and number of platelets, respectively. Labeled platelets (5.4×10^7) of either genotype were infused as bolus via the venous catheter.

Intravital Microscopy after Intestinal I/R. To evaluate the biological significance of cGKI in the regulation of platelet adhesion/aggregation in vivo, fluorescent platelets were infused after intestinal I/R and visualized in the postischemic microcirculation by intravital fluorescence microscopy. 4–6-wk-old inbred 129sv mice of either genotype (five to nine litter- or age-matched animals per group) were anesthetized by inhalation of isoflurane-N_2O (0.35 FiO_2, 0.015 liter/liter isoflurane; Forene®; Abbott GmbH). The animals were placed on a heating pad (Effenberger), and polyethylene catheters (Portex) were implanted into the left carotid artery and left jugular vein for continuous recording of mean arterial blood pressure and infusion of fluorescent platelets, respectively. After laparotomy, a segment of the jejunum was exteriorized and constantly superfused with 37°C Ringer's lactate. Segmental jejunal ischemia was induced for 60 min by occluding the supplying vessels with microsurgical clips. After reperfusion, the intestinal segment was exposed on a mechanical stage and platelet–platelet and platelet–endothelial cell interactions in the postischemic microvasculature were investigated by intravital microscopy. 15 min after the onset of the reperfusion, labeled platelets (5.4×10^7) of either genotype were infused as bolus via the venous catheter into the acceptor mouse of either genotype subjected to either I/R or sham operation. Platelet concentration in wild-type and $cGKI^{-/-}$ mice was $0.5 \pm 0.02 \times 10^6$ ($n = 36$) and $0.5 \pm 0.06 \times 10^6$ ($n = 25$)/μl blood, respectively. During the reperfusion, 10 nonoverlapping regions of interest from the submucosal vessels of the ischemic/reperfused segment were randomly selected in each mouse and observed for 30 s with a modified microscope (Leitz). The microscopic images with a final magnification on the video screen of 450× were recorded by a CCD camera (FK6990, Cohu; Prospective Measurements) connected to a video recording system (Sony Corp.). For analysis of platelet–platelet and platelet–endothelial cell interactions, a computer-assisted image analysis program (CAP IMAGE; Dr. Zeintl, University of Heidelberg, Heidelberg, Germany) and frame-to-frame analysis of the videotapes were used (23). All experiments were blinded, and adherence of platelets to the surface of arterioles and venules (vessel diameter, 15–85 μm) and formation of platelet aggregates in capillaries (diameter, 15 μm), arterioles, and venules (vessel diameter, 15–85 μm) were quantified. The number of adherent platelets was

assessed by counting the platelets that did not move or detach from the endothelial surface within 15 s. Platelet adhesion is presented per square millimeter of endothelial surface. The number of occluding and nonoccluding aggregates was quantified within arterioles and venules and is presented per 100 vessels. To determine platelet aggregation in the capillary bed, the length (centimeter) of capillaries occluded by fluorescent platelets was measured and calculated per square centimeter of tissue cross-sectional area.

Preparation of Platelets for Renal I/R. To confirm the role of cGKI in the regulation of I/R-induced platelet adhesion/aggregation in vivo, the accumulation of ^{111}I-labeled wild-type or cGKI$^{-/-}$ platelets was assessed in the postischemic kidney. Platelets were pelleted from PRP (see above) and resuspended in 500 μl PBS (Ca^{2+} and Mg^{2+} free) containing 37 MBq/ml ^{111}I-oxine (Amersham Healthcare). After incubation for 5 min at 37°C, the platelet suspension was centrifuged for 10 min at 1,000 *g* and the supernatant was removed. The resulting pellet was washed with 2 ml PBS (Ca^{2+} and Mg^{2+} free) and centrifuged for 10 min at 1,000 *g*. After removing the supernatant, the pellet was resuspended with 200 μl PBS (Ca^{2+} and Mg^{2+} free).

I/R of the Kidney. Male wild-type 129sv mice were anesthetized using intraperitoneal injection of Avertin (1.2% tribromoethanol/amylalcohol in 0.9% saline solution) and placed on a heating pad for maintenance of body temperature. A polyethylene catheter (Portex) was implanted into the left jugular vein for infusion of ^{111}I-labeled platelets and for infusion of Ringer's lactate to maintain euvolemia. After a midline incision of the abdomen, the left renal artery and vein were isolated and subsequently occluded with a microsurgical clip for 30 min. 25 min after setting occlusion, 0.2 ml of washed, ^{111}I-labeled platelet suspension (2×10^7) of either genotype was infused via the venous catheter, and 5 min afterwards the clip was removed for reperfusion. After 25 min of reperfusion, the experiment was terminated and the kidney was removed, weighed, and homogenized. The homogenate was counted (Tri-carb 2100 TR; Packard Instrument Co.), and the accumulation of ^{111}I-labeled wild-type and cGKI$^{-/-}$ platelets after renal I/R was quantified as counts per minute per milligram wet weight of the kidney (24).

Preparation of Platelets for Flow Cytometry. To study the role of cGKI in the regulation of fibrinogen binding to agonist-stimulated wild-type and cGKI$^{-/-}$ platelets, blood (0.4–0.6 ml) was collected from wild-type ($n = 7$) and cGKI$^{-/-}$ ($n = 5$) mice by cardiac puncture. The platelets were separated as described above for intravital microscopy, with the exception that no rhodamine was added. The resultant pellet was resuspended in 6 ml of a solution containing equal parts PBS with and without Ca^{2+} and Mg^{2+} (PAN Systems). The purity and number of platelets were determined using the A^C T counter.

Assessment of Platelet Fibrinogen Binding In Vitro. The wild-type or cGKI$^{-/-}$ platelets were preincubated at room temperature for 2 min with either PBS (PAN Systems), the NO-donor DEA-NO (100 nM final concentration; Alexis) or the stabile prostacyclin-analogue iloprost (10 μM final concentration, Ilomedin; Schering AG). After preincubation, the samples were stimulated with 0.2 U/ml mouse thrombin (Sigma-Aldrich) or PBS, and immediately incubated for 10 min at room temperature with Alexa™ 488-conjugated fibrinogen (12.5 μg/ml final concentration; Molecular Probes). After incubation, all samples were fixed with 1% paraformaldehyde and the fluorescence intensity was analyzed using a flow cytometer (FACSort®; excitation at 488 nm, emission detection at 520 nm). The platelets were identified by their characteristic forward and sideward light scatter. Analysis of the fluorescence properties of 10,000 platelets was performed using a Lysis II data handling program (Becton Dickinson). The fluorescence intensity of unstimulated platelets, preincubated with DEA-NO, iloprost, or PBS (<15% of thrombin-stimulated fluorescence intensity), was subtracted from the fluorescence of the corresponding thrombin-stimulated sample. Data are presented as percentage of nonpretreated, thrombin-stimulated wild-type or cGKI$^{-/-}$ platelets.

Statistical Analysis. All data are presented as mean ± SEM. Statistical differences between two means were determined by Student's *t* test or Kruskal-Wallis test (see figures). $P < 0.05$ was regarded as significant, *n* indicates the number of animals.

Results and Discussion

cGKI$^{-/-}$ Platelets Are Unresponsive to cGMP In Vitro. Deletion of the cGKI gene abolished immunoreactive cGKI protein without affecting the immunoreactive concentration of cAMP kinase (Fig. 1 A). Platelet activation in response to collagen was similar in wild-type and cGKI$^{-/-}$ mice (Fig. 1, C–G). In both wild-type and mutant platelets, collagen-induced shape change, aggregation, and serotonin release were prevented by activation of cAK by the cAK-specific cAMP-analogue cBIMPS (Fig. 1, C–G). In contrast, activation of cGKI by the cGMP-analogue 8-pCPT-cGMP inhibited collagen-induced shape change, aggregation, and serotonin release in wild-type platelets, but had no effects on cGKI$^{-/-}$ platelets. This indicates that the effects of cGMP are mediated predominantly via activation of cGKI, whereas other cGMP receptors, such as phosphodiesterases and cyclic nucleotide-gated ion channels, play a minor role in the cGMP-dependent inhibition of platelet aggregation in vitro. Therefore, it is very likely that NO, which is known to elevate platelet cGMP levels (5), inhibits platelet adhesion and aggregation acting via cGKI.

Cross-activation of cAK by cGMP does not appear to be operative in platelets. The reverse mechanism (i.e., activation of cGKI by cAMP, which has been postulated to mediate relaxation of smooth muscle cells; reference 25) is also not involved in the regulation of platelet function since the specific activator of cAK, cBIMPS, elicited a similar effect on both cGKI$^{+/+}$ and cGKI$^{-/-}$ platelets. We therefore conclude that cGMP and cAMP signaling cascades inhibit platelet aggregation independent from each other.

VASP Phosphorylation Is Defective in Platelets Lacking cGKI. Several mechanisms might be involved in cGMP/cGKI-dependent inhibition of platelet aggregation. In various cell types, including platelets and smooth muscle cells, cGMP/cGK lowers cytosolic Ca^{2+} concentrations after stimulation, thereby affecting a variety of Ca^{2+}-regulated processes (25–28). Yet the details of cGMP/cGK-dependent regulation of platelet Ca^{2+} homeostasis remain to be elucidated at the molecular level. Phosphorylation processes are likely to be involved in the antiaggregatory effects of cGMP/cGKI on platelets.

To date, the only well-established substrate of cGK is VASP, a 46–50-kD vasodilator-stimulated protein, present in high concentrations in platelets (29). In the study reported here, VASP phosphorylation was assessed in wild-

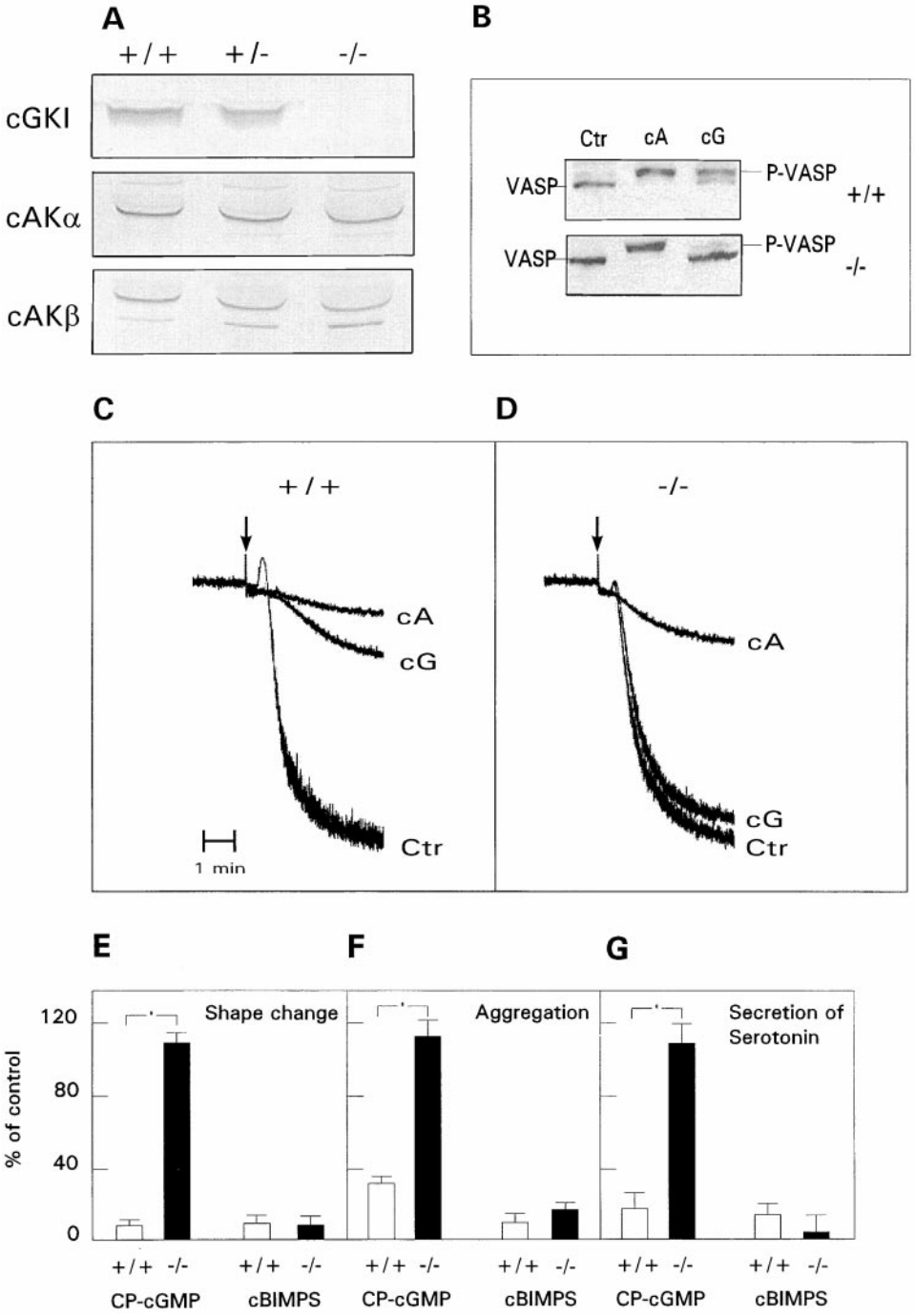

Figure 1. cGKI deletion affects platelet function. (A) Western blot analysis of cGKI and catalytic subunits of cAKα and cAKβ in platelets of wild-type (+/+), heterozygous (+/−), and cGKI-deficient (−/−) mice. (B) Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in platelets in the absence (Ctr) and presence of 100 μM 8-pCPT-cGMP (cG) or cBIMPS (cA). (C–G) Effect of 100 μM 8-pCPT-cGMP (cG) and 100 μM cBIMPS (cA) on collagen-induced platelet aggregation. Representative traces of shape change and aggregation of wild-type (C) and cGKI$^{-/-}$ (D) platelets. The arrow indicates the addition of collagen (5 μg/ml). Summary of the effects of 8-pCPT-cGMP (CP-cGMP) and cBIMPS on shape change (E), aggregation (F), and secretion of 5-hydroxytryptamine (G) in cGKI$^{+/+}$ and cGKI$^{-/-}$ platelets. Values are presented as mean ± SEM of four to six animals. *$P < 0.05$, Student's t test.

type and cGKI$^{-/-}$ platelets using the mobility shift of phosphoVASP (9). In murine platelets, VASP is phosphorylated in response to both cBIMPS and 8-pCPT-cGMP (Fig. 1 B). The disruption of cGKI abolished cGMP-dependent in vivo phosphorylation of VASP in platelets without affecting VASP phosphorylation in response to the cAMP analogue cBIMPS. Although VASP phosphorylation correlates well with platelet inhibition, its precise functional role has not been established thus far. However, the subcellular location of VASP and the association with actin filaments and focal adhesions suggest a possible role in regulating platelet aggregation/adhesion (30). In fact, there is evidence to suggest that VASP phosphorylation is closely associated with the inhibition of the agonist-evoked activation of the fibrinogen-binding site of the glycoprotein IIb–IIIa (31, 32), supporting a role of cGKI/VASP signaling in the regulation of platelet adhesion/aggregation.

cGKI Attenuates the Adhesion and Aggregation of Platelets within the Postischemic Intestine. Growing evidence suggests that platelets play an important role in the pathogenesis of I/R-induced vascular injury and restenosis. Ischemia is associated with platelet accumulation early after the onset of

reperfusion (17). Clinical and experimental studies have indicated that platelet adhesion and aggregate formation and the release of proinflammatory mediators from activated platelets in response to ischemia may impair restoration of nutritive blood supply during reperfusion (33, 34). Although activation of cGKI attenuates platelet aggregation in vitro, the significance of the NO/cGMP/cGKI pathway in the homeostasis of platelet adhesion/aggregation during I/R in vivo has not been clearly defined thus far. The biological role of cGKI might be questioned, since the deficit in cGKI could be compensated by endothelium-derived prostacyclin signaling through the unperturbed cAK pathway (12). Therefore, to study the physiological relevance of cGKI in vivo, we determined platelet adherence and aggregation within the microcirculation of an ischemic/reperfused segment of the jejunum using intravital video microscopy. Wild-type and $cGKI^{-/-}$ mice (acceptor) were subjected to intestinal ischemia (60 min). After reperfusion, fluorescent wild-type and $cGKI^{-/-}$ platelets were infused and visualized within the intestinal submucosa (arterioles, capillaries, and venules) by intravital fluorescence microscopy.

Since platelet adhesion to the injured vessel surface represents an early step in the process of platelet accumulation/aggregation, we first determined the number of adherent platelets within arterioles and postcapillary venules of the postischemic submucosal microcirculation. Under control conditions without I/R (sham), wild-type platelets did not interact with wild-type endothelium (Fig. 2). In contrast, numerous platelets were found firmly attached to the vascular wall of both arterioles and postcapillary venules in response to I/R (45 ± 18 and 43 ± 24 adherent platelets/mm^2, respectively; Figs. 2 and 3, A and B). To assess the role of cGKI in the regulation of platelet function in vivo, $cGK^{-/-}$ platelets were transfused into cGK-deficient mice after I/R. The loss of cGKI drastically enhanced postischemic platelet–endothelial cell interactions. Within both arterioles and venules, the number of adherent platelets was increased four- to sixfold when compared with wild-type animals. Under control conditions, no adhesion of $cGKI^{-/-}$ platelets to the vessel wall was observed, indicating that a pathological stimulus, such as I/R, is required to induce platelet adhesion in $cGKI^{-/-}$ mice.

Because platelets, endothelial cells, and smooth muscle cells express cGKI, we wanted to clarify whether cGKI expressed by platelets or present in the vascular wall is necessary to inhibit platelet adhesion during postischemic reperfusion. To determine the contribution of platelet cGKI, $cGKI^{-/-}$ platelets were infused into wild-type mice. The isolated loss of platelet cGKI dramatically enhanced I/R-induced platelet adhesion to the vascular wall of wild-type animals: 290 ± 45 and 181 ± 28 platelets were found firmly attached per square millimeter endothelial surface of arterioles and venules, respectively (Figs. 2 and 3, A and B). Therefore, platelet adhesion is enhanced to a similar extent, independent of whether platelets alone or both platelets and vascular wall lack cGKI. Accordingly, I/R-induced adhesion of wild-type platelets was not increased in cGKI null mutants as compared with wild-type recipients (Figs. 2

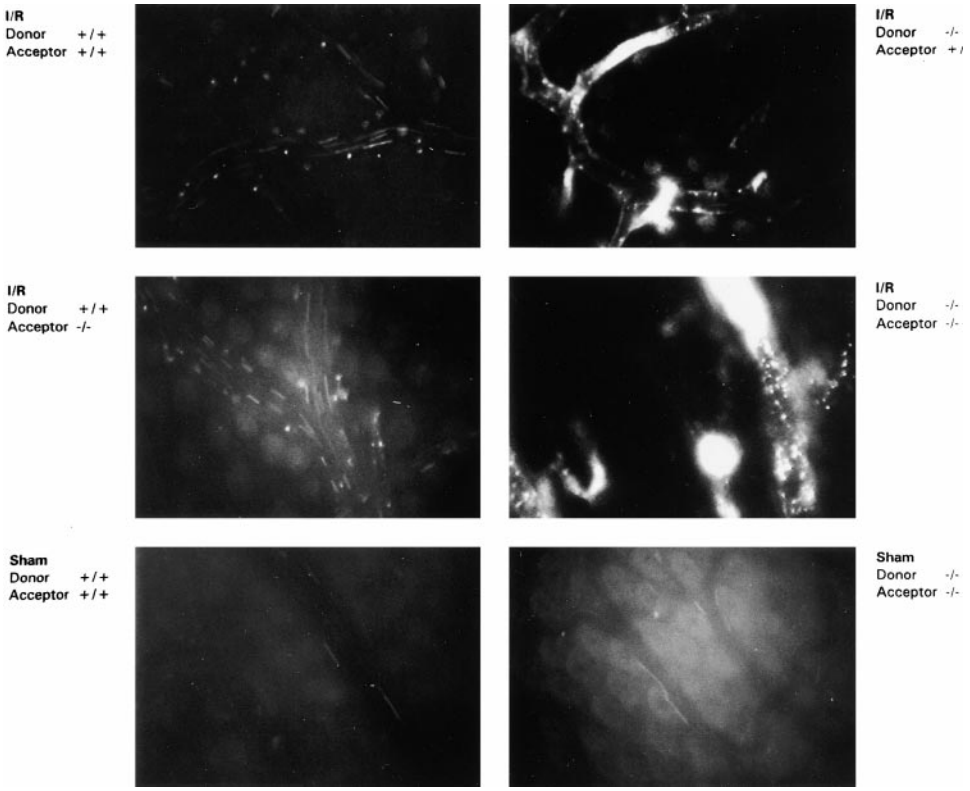

Figure 2. Photomicrographs of the postischemic intestinal microcirculation. Platelet–endothelial cell and platelet–platelet interactions are visualized by intravital fluorescence microscopy. Representative pictures from postischemic submucosal arterioles are presented. Sham-operated (sham) animals without I/R served as controls. Donor, animal genotype of the donor platelets; acceptor, genotype of the recipient, subjected to I/R.

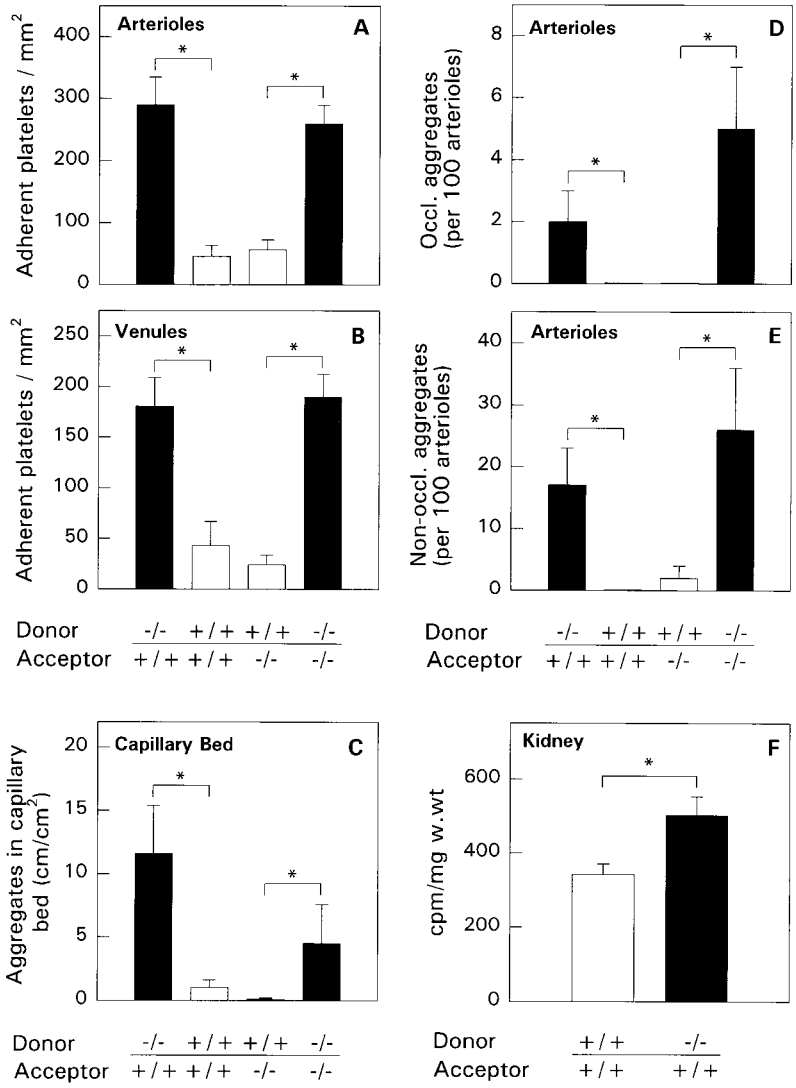

Figure 3. Summary of ischemia–reperfusion experiments. The adherent platelets in arterioles (A) and venules (B) were quantified and defined as platelets that did not move or detach from the endothelial surface within 15 s. The number of adherent platelets per square millimeter of endothelial surface, calculated from the diameter and length of the observed vessel segment, is presented. (C) Aggregates in capillaries (diameter < 10 μm) were quantified as length of occluded capillaries (centimeters) per square centimeter tissue cross-sectional area. (D) Occluding and (E) nonoccluding platelet aggregates per 100 arterioles (diameter, 15 μm) of a postischemic segment of the jejunum. (F) Accumulation of ^{111}Indium-labeled platelets in the kidney after I/R. All values are mean ± SEM, derived from five to nine animals per group. $^*P < 0.05$ by the Kruskal-Wallis test.

and 3 A). This demonstrates that the cGKI expressed in endothelium and smooth muscle cells plays a minor role in the regulation of platelet adhesion dynamics during I/R.

During reperfusion, adherent platelets may subsequently aggregate leading to luminal narrowing and complete vascular (re-)occlusion, resulting in additional ischemia of the supplied tissue (18–20). To evaluate the participation of cGKI in the regulation of platelet aggregation in vivo, we quantified the presence of platelet aggregates in arterioles and venules of the postischemic jejunal segment. The number of occluding and nonoccluding aggregates in arterioles and venules was assessed within 10 randomly selected regions of interest and is presented per 100 vessels (Figs. 2 and 3, D and E). Whereas aggregation of wild-type or cGKI$^{-/-}$ platelets was virtually absent under control conditions, platelets deficient in cGKI showed a very strong tendency to aggregate in response to I/R. In postischemic arterioles, 2–5 occluding and 17–26 nonoccluding aggregates were observed per 100 vessels when cGKI$^{-/-}$ platelets were transfused into wild-type or cGKI$^{-/-}$ mice, respectively (Fig. 3, D and E). Likewise, the absence of cGKI in platelets enhanced aggregation in postcapillary venules (not shown). In contrast, no occluding or nonoccluding aggregates were detected when both platelets and the vascular wall expressed cGKI (Fig. 3, D and E), indicating that cGMP/cGKI signaling pathways regulate both platelet adhesion and aggregation. To assess the role of cGKI present in endothelial cells and vascular smooth muscle, wild-type platelets were transfused into cGKI$^{-/-}$ mice (Fig. 3, D and E). However, the absence of cGKI in the vascular wall did not significantly enhance the aggregation of wild-type platelets in arterioles or venules, suggesting that endothelial cell and smooth muscle cGKI are not involved in the regulation of platelet aggregation in vivo.

Platelet aggregation in cGKI mutants was not confined to arterioles and venules, but was also frequently observed in capillaries (diameter, 10 μm). To assess the extent of platelet aggregation in the capillary bed of the postischemic submucosa, the length (centimeters) of capillaries occluded by fluorescent platelets was measured and calculated per square

centimeter tissue cross-sectional area (Figs. 2 and 3 C). Platelet aggregates in capillaries were nearly absent after transfusion of wild-type platelets into wild-type mice or cGKI mutants. In contrast, aggregation of $cGKI^{-/-}$ platelets in postischemic capillaries was a prominent phenomenon, independent of whether or not the vascular wall expressed cGKI.

Hence, platelet adhesion and platelet aggregation in arterioles, capillaries, and venules are drastically enhanced when the platelets lack cGKI. In contrast, the absence of the endothelial/smooth muscle cGKI has no significant effects on homotypic platelet–platelet or heterotypic platelet–endothelium interactions in response to I/R. This indicates that under pathophysiological conditions, such as I/R, NO acts via the cGKI present in platelets to regulate platelet adhesion and aggregation in vivo. Although endothelial cells are considered to be the major source of NO, in particular under conditions associated with platelet activation, platelet-derived NO also plays an important role in regulating platelet aggregation and platelet recruitment (35). During aggregation, the NO release by platelets is significantly increased (35). This increase in NO formation can act via guanylyl cyclase to activate platelet cGKI. Therefore, platelets appear to have the ability to self-regulate their adhesion and aggregation upon activation by an autocrine/paracrine mechanism in which activated platelets release NO that acts on platelet cGKI to attenuate both adhesion and aggregation.

It appears noteworthy that, under physiological conditions without I/R, platelet aggregation in arterioles, capillaries, and venules was absent in both wild-type animals and $cGKI^{-/-}$ mutants (not shown). This indicates that both endothelial cells and platelets are in an antiadhesive/anticoagulant state under physiological conditions and acquire a proadhesive/procoagulant phenotype in response to I/R.

Increased Platelet Accumulation after Renal I/R In Vivo. To evaluate whether the observed increase in postischemic platelet adhesion and aggregation due to the loss of platelet cGKI is confined to the intestine or rather a more general defect in platelet function, we analyzed the accumulation of wild-type and $cGKI^{-/-}$ platelets after I/R of the kidney. Wild-type animals were laparotomized, and the left renal artery and vein were cross-clamped for 30 min using microsurgical clips. 5 min before reperfusion, washed ^{111}I-labeled platelets were infused via a catheter implanted into the left jugular vein. After 25 min of reperfusion, the left kidney was excised and platelet accumulation was quantified as counts per minute per milligram wet weight. Renal I/R induced the accumulation of ^{111}Indium-labeled wild-type platelets in the postischemic wild-type kidney. However, platelets lacking cGKI exhibited a 46% higher accumulation in the wild-type kidney after I/R when compared with wild-type platelets (Fig. 3 F; $P < 0.05$), suggesting that cGKI inhibits postischemic platelet accumulation independent of the organ studied.

cGKI Inhibits Fibrinogen Binding to Thrombin-stimulated Platelets. The molecular mechanisms underlying cGKI-dependent inhibition of platelet adhesion/aggregation during I/R in vivo are as yet unclear. There is growing evidence indicating that I/R is associated with an activation of the platelet fibrinogen receptor, the glycoprotein (GP) IIb–IIIa (36). Binding of fibrinogen to the activated form of the platelet GP IIb–IIIa integrin complex plays a critical role in the process of platelet adhesion/aggregation (37, 38). Although both NO and cGMP have been shown to interfere with agonist-evoked activation of the GP IIb–IIIa (31, 39), the exact role of cGKI in the inhibition of GP IIb–IIIa activation has not been identified thus far. To determine whether cGKI mediates NO/cGMP-dependent regulation of GP IIb–IIIa function, we have investigated the effects of NO on agonist-induced fibrinogen binding to wild-type and $cGKI^{-/-}$ platelets in vitro. In wild-type platelets, pretreatment with NO decreased thrombin-induced fibrinogen binding by ~46% (Fig. 4). In contrast, in platelets lacking cGKI, the response to NO was nearly absent ($P < 0.05$). Hence, cGKI-dependent inhibition of the GP IIb–IIIa adhesion complex is involved in the regulation of platelet adhesion/aggregation by NO/cGMP in vivo. The loss of platelet cGKI does not affect the ability of platelets to respond to prostacyclin. Preincubation with iloprost reduced agonist-induced fibrinogen binding to both wild-type and $cGKI^{-/-}$ platelets by ~40%. Therefore, the inability of the endogenous cAMP kinase-activating system to compensate for the loss of cGKI in vivo is not due to alterations in the response of $cGKI^{-/-}$ platelets to exogenous prostacyclin.

In conclusion, we have demonstrated that platelet cGKI attenuates agonist-induced platelet activation, fibrinogen

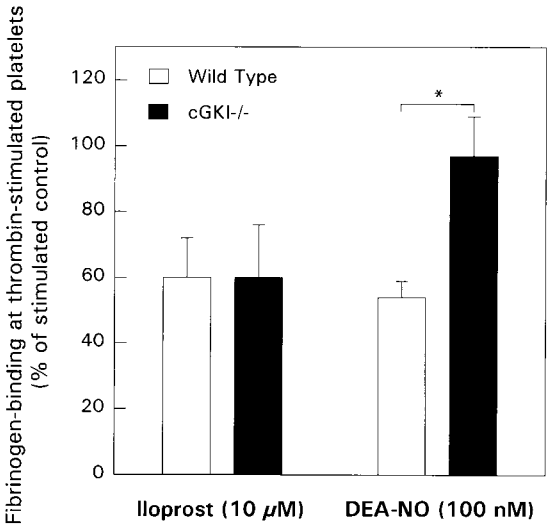

Figure 4. Summary of flow cytometric experiments. Platelet samples, preincubated with iloprost or DEA-NO, were stimulated with mouse thrombin or PBS in the presence of Alexa™ 488–conjugated fibrinogen. Binding of the fluorescent fibrinogen to wild-type and $cGKI^{-/-}$ platelets was assessed by flow cytometry. The fluorescence intensity obtained from nonstimulated platelets pretreated with PBS, DEA-NO, or iloprost was subtracted from the fluorescence of thrombin-stimulated platelets, preincubated with PBS, DEA-NO, or iloprost, respectively. Fibrinogen-binding to thrombin-stimulated wild-type or $cGKI^{-/-}$ platelets in the presence of prostacyclin or DEA-NO was calculated as percentage of the nonpretreated, thrombin-stimulated control sample. All values are mean ± SEM, derived from five to seven animals per genotype. *$P < 0.05$ by the Student's *t* test.

binding, and aggregation in vitro. Moreover, the loss of cGKI in platelets is associated (a) with an increase in platelet accumulation in the postischemic kidney and (b) with a significant enhancement of both platelet adhesion and aggregation in the postischemic intestinal microvasculature in vivo. The platelet cAMP/cAK signaling cascade does not compensate for the loss of cGKI under pathophysiological conditions. Platelets are known to release NO upon activation; therefore, an autocrine/paracrine signaling cascade, including platelet-derived NO and platelet cGKI, is likely to be involved in the regulation of platelet adhesion and aggregation under physiological and, in particular, pathophysiological conditions. Since the accumulation and aggregation of platelets after endothelial injury is a common pathophysiological mechanism underlying many of the most important diseases, including myocardial infarction, angina pectoris, thrombotic stroke, and peripheral vascular insufficiency, specific activators of platelet cGKI might present a powerful strategy aimed at the prevention of I/R injury.

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