Mechanism of platelet functional changes and effects of anti-platelet agents on in vivo hemostasis under different gravity conditions

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Li S, Shi Q, Liu G, Zhang W, Wang Z, Wang Y, Dai K. Mechanism of platelet functional changes and effects of anti-platelet agents on in vivo hemostasis under different gravity conditions. J Appl Physiol 108: 1241–1249, 2010. First published February 4, 2010; doi:10.1152/japplphysiol.01209.2009.—Serious thrombotic and hemorrhagic problems or even fatalities evoked by either microgravity or hypergravity occur commonly in the world. We recently reported that platelet functions are inhibited in microgravity environments and activated under high-G conditions, which reveals the pathogenesis for gravity change-related hemorrhagic and thrombotic diseases. However, the mechanisms of platelet functional variations under different gravity conditions remain unclear. In this study we show that the amount of filamin A coimmunoprecipitated with GPIb was enhanced in platelets exposed to modeled microgravity and, in contrast, was reduced in 8 G-exposed platelets. Hypergravity induced actin filament formation and redistribution, whereas actin filaments were reduced in platelets treated with modeled microgravity. Furthermore, intracellular Ca2+ levels were elevated by hypergravity. Pretreatment of platelets with the cell-permeable Ca2+ chelator BAPTA-AM had no effect on cytoskeleton reorganization induced by hypergravity but significantly reduced platelet aggregation induced by ristocetin/hypergravity. Two anti-platelet agents, aspirin and tirofiban, effectively reversed the shortened tail bleeding time and reduced the death rate of mice exposed to hypergravity. Furthermore, the increased P-selectin surface expression was obviously reduced in platelets from mice treated with aspirin/hypergravity compared with those from mice treated with hypergravity alone. These data suggest that the actin cytoskeleton reorganization and intracellular Ca2+ level play key roles in the regulation of platelet functions in different gravitational environments. The results with anti-platelet agents not only further confirm the activation of platelets in vivo but also suggest a therapeutic potential for hypergravity-induced thrombotic diseases.

platelets; cytoskeleton; calcium; modeled microgravity; hypergravity

GRAVITATIONAL CHANGES induce pathophysiological responses in various cell types such as vascular endothelial cells (18), neuroblastoma cells (30), and bone cells (34). Many thrombotic and hemorrhagic diseases have been documented in human beings exposed to microgravity or hypergravity environments (27, 32), and several possibly related organs have been examined to explore the mechanisms underlying these gravity change-related diseases (28, 39). We reported recently that platelet functions are inhibited in microgravity environments and activated under high-G conditions, which reveals the pathogenesis of gravity change-related hemorrhagic and thrombotic diseases (8, 11, 19). However, the mechanisms of the platelet functional variations under different gravity conditions remain unclear.

The actin cytoskeleton of cells is a highly dynamic structure and is sensitive to changes of gravity conditions (30, 38). Several studies have shown that simulated hypogravity stimulates endothelial cell growth and also remodels the actin cytoskeleton and reduces the total amount of actin (1, 38). In contrast, hypergravity alters the distribution of actin fibers without affecting the total amount of actin (38). In human mesenchymal stem cells, F-actin stress fibers are disrupted within 3 h of initiation of modeled microgravity and are completely absent by 7 days, whereas monomeric G-actin is increased (22). Furthermore, studies in Biorack on space shuttle missions STS-76, STS-81, and STS-84 confirmed the collapse of the actin cytoskeleton in cells grown in microgravity conditions. However, flown cells under I-G conditions maintained normal actin cytoskeleton and fibronectin matrix (14). In addition, experiments on single-cell behavior in hypergravity have shown that hypergravity stimulation results in reorganization of actin fibers with an increased formation of dense actin fibers in cytosol (18). We have reported that glycoprotein (GP) Ibα surface expression and association with the cytoskeleton significantly decreased in platelets exposed to simulated microgravity and obviously increased in hypergravity-exposed platelets (8, 11), indicating that the reorganization of cytoskeleton may be involved in the regulation of platelet functional changes under different gravity conditions.

Changes in the intracellular Ca2+ concentration ([Ca2+]i) in response to altered gravity have been reported to occur widely in many types of cells and to associate with cytoskeletal reorganization (4, 36). [Ca2+]i increases in response to gravity change in hypocotyls and petioles of Arabidopsis seedlings, which depends on both Ca2+ influx and Ca2+ release from intracellular Ca2+ stores (35). The elevation of [Ca2+]i is sufficient for promoting cytoskeletal reorganization (25); on the other hand, dynamic remodeling of the actin cytoskeleton also modulates intracellular Ca2+ release (4, 35). The intracellular Ca2+ concentration plays key roles in initiating and regulating platelet adhesion, aggregation, and secretion (21), thus raising the concern whether platelet functional changes under different gravitational conditions result from variations of [Ca2+]i.

In this study, we show that the association of GPIbα with filamin A and the organization of actin cytoskeleton are regulated by different gravities. Intracellular Ca2+ levels were elevated by hypergravity, which upregulated ristocetin-induced platelet aggregation. Furthermore, two anti-platelet agents, aspirin

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and tirofiban, effectively reversed platelet activation induced by hypergravity in vivo and reduced the death rate of mice exposed to hypergravity.

**MATERIALS AND METHODS**

Reagents. Monoclonal antibody SZ2 against GPIbα was kindly provided by Dr. Changgeng Ruan (Soochow University, Suzhou, China). Monoclonal antibody against filamin A was purchased from Novocastra Laboratories (Newcastle, UK). Polyclonal goat anti-human P-selectin antibody sc-6941 and FITC-labeled rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ristocetin, ADP, EDTA, and EGTA were purchased from Sigma (St. Louis, MO). 1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) was purchased from Dojindo Molecular Technologies (Rockville, MD). The rhodamine-conjugated phalloidin and fluo-3 AM were obtained from Invitrogen Molecular Probes (Eugene, OR).

**Rotary cell culture system.** To simulate microgravity conditions, we used a National Aeronautics and Space Administration-recommended and widely accepted ground-based microgravity-simulating system, the Rotary Cell Culture System (RCCS; Synthecon, Houston, TX) (5, 21). Platelets were either rotated about a horizontal axis perpendicular to the gravitational vector to randomize gravitational vectors across the surface of the platelets and generate microgravity of \( \sim 10^{-2} \) g or rotated about a vertical axis parallel to the gravitational vector to experience normal gravitational forces and serve as a control (1 G) environment (5, 12).

**Hypergravity stimulation.** Hypergravity was applied to platelets or mice with a custom-built slow-speed 30-cm-radius centrifuge. Platelets or mice were placed on the plate of the centrifuge and rotated for a certain time at 253 or 311 rpm to obtain 8 or 12 G, respectively, at room temperature (RT). The mice were anesthetized with pentobarbital sodium before centrifugation to avoid mechanical injury. The synchronous 1-G controls were placed on a plateau for 15 min. The 1-G control mice injected with 4- to 6-wk-old adult C57 black or BALB/C mice were anesthetized with an intraperitoneal injection of pentobarbital sodium. To study the effects of aspirin and tirofiban treatments, anesthetized mice were intraperitoneally injected with aspirin (170 mg/kg) and caudally intravenously injected with tirofiban at a dose of 0.4 mg/kg, respectively, and then exposed to 12-G hypergravity for 15 min. Alcohol was used as a control for aspirin; 0.15 M NaCl was used as a control for tirofiban. The synchronous 1-G control mice that were matched for weight and sex were treated in the same way and then placed on a plateau for 15 min. The 1-G control mice injected with aspirin or tirofiban were used to confirm the rationality of drug dose used that would not cause spontaneous bleeding in mice. All procedures performed in this study were approved by the Peking University Institutional Animal Care and Use Committee.

**Preparation of washed platelets and platelet-rich plasma.** For mouse experiments, fresh blood was immediately drawn from the inferior caval vein of mice after 12- or 1-G exposure and anticoagulated with a 1:7 volume of ACD. An equal volume of 4% phosphate-buffered paraformaldehyde was then added to incubate for 30 min at RT. After centrifugation, isolated platelets were washed twice with CGS and then resuspended in modified Tyrode’s buffer to a concentration of 1 \( \times 10^{10} \) platelets/ml (7).

**Coimmunoprecipitation and immunoblotting.** Washed human platelets (3 \( \times 10^{8} \) platelets/ml) were exposed to simulated microgravity for 30 min or 1 h, 8 G for 10 or 15 min, or synchronous 1-G conditions and then lysed with buffer containing 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1:100 aprotinin on ice for 30 min. Platelet lysates were centrifuged at 15,000 g for 4 min to remove cytoplasmic actin filaments. The supernatants were immunoprecipitated with anti-GPIbα NH2-terminal antibody SZ2 and protein G-Sepharose beads. Samples were subjected to SDS-PAGE and immunoblotted with SZ2 and anti-filamin A antibody, respectively. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL).

**Staining of platelet F-actin.** Washed human platelets (3 \( \times 10^{8} \) platelets/ml) were exposed to simulated microgravity for 1 h, 8 G for 15 min, or synchronous 1-G conditions and immediately fixed with 4% paraformaldehyde. F-actin was stained by rhodamine-conjugated phalloidin according to the previously reported method (18). To evaluate the effect of the intracellular Ca2+ chelator BAPTA-AM on actin distribution under hypergravity, platelets were pretreated with BAPTA-AM at 10 μM at 37°C for 20 min, centrifuged at 8 G for 15 min, and then stained. As a negative control, platelets were pretreated with vehicle DMSO.

**Measurement of intracellular Ca2+ concentration.** Intracellular Ca2+ levels were determined with the Ca2+-sensitive fluorescent dye fluo-3 AM using flow cytometry. Briefly, washed human platelets (3 \( \times 10^{8} \) platelets/ml) were loaded with 8 μM fluo-3 AM for 30 min at 37°C in the dark. After being washed once, platelets were resuspended at a concentration of 5 \( \times 10^{8} \) platelets/ml. The external Ca2+ was adjusted to 1 mM, and then the dyed platelets were exposed to simulated microgravity for 1 h, 8 G for 15 min, or synchronous 1 G and analyzed using flow cytometry. In some experiments, the loaded platelets were preincubated with 5 mM EDTA (or EGTA) at RT for 10 min and then centrifuged at 8 G for 15 min and analyzed.

**Measurement of platelet aggregation.** Platelet aggregation was performed using a turbidometric platelet aggregometer (Xinpusen, Beijing, China). Briefly, PRP was exposed to 8 or 1 G for 15 min and then immediately added into glass aggregometer cuvettes, and aggregation was induced by addition of the agonist ristocetin (0.6–0.8 mg/ml) or ADP at 37°C under stirring (1,000 rpm) conditions. To evaluate the effect of BAPTA-AM on platelet aggregation, PRP was preincubated with BAPTA-AM (10 μM) at 37°C for 20 min before hypergravity or 1-G treatment, and then aggregation was measured. Vehicle DMSO was used as a negative control.

**Tail bleeding time assay.** Tail bleeding time assays were performed with 4- to 6-wk-old adult C57 black and BALB/C mice. After application of hypergravity at 12 G for 15 min, the differently treated animals and 1-G control mice were immediately subjected to tail bleeding time analysis. The tails were cut transversely 0.5 mm from the tip and immersed in 0.15 M NaCl maintained at 37°C. The time to arrest of bleeding was noted. The occurrence of rebleeding from tail wounds was assessed. Maximum bleeding times allowed were 10 min after the tail was cauterized.

**Platelet surface staining.** Washed mouse platelets (1 \( \times 10^{7} \) platelets/ml) were incubated with goat anti-mouse P-selectin antibody at RT for 30 min. Platelets were washed once and further incubated with 10 μg/ml FITC-labeled rabbit anti-goat IgG for 30 min at RT in the dark. Samples were analyzed using flow cytometry, and antibody binding was expressed as geometric mean fluorescence intensity. As negative controls, platelets were incubated with goat IgG and then incubated with FITC-labeled rabbit anti-goat IgG.
Statistical analysis. The Western blot was scanned, and the optical density of each band from the same blot was quantitated with NIH ImageJ software (image processing and analysis in Java; National Institutes of Health). Data are means ± SD. Statistical significance between means was determined by using Student’s t-test with InStat software. A P value <0.05 was considered significant.

RESULTS

Effects of simulated microgravity and hypergravity on GPIbα-filamin A interaction. Filamin A is a large actin-binding protein that stabilizes actin network and links GPIb-IX complex to the cytoskeleton via binding to the cytoplasmic domain of GPIbα (26). Our previous data indicated that GPIbα was obviously detached from the cytoskeleton and presented in soluble components of platelets treated with modeled microgravity. In contrast, more GPIbα was attached to the cytoskeleton under hypergravity conditions, suggesting that the altered association of GPIbα with the cytoskeleton in response to different gravity conditions plays a role in the variations of platelet function (8, 11). To explore the mechanisms underlying the effect of gravity on the association of GPIbα with the cytoskeleton, washed human platelets were treated with RCCS to simulate microgravity or centrifuged with a special low-speed centrifuge to produce 8-G force, and then filamin A was immunoprecipitated with GPIbα from the soluble components of platelet lysates. As shown in Fig. 1, filamin A was time-dependently coimmunoprecipitated with GPIbα in platelets treated with modulated microgravity, in contrast, the amount of filamin A coimmunoprecipitated with GPIbα was time-dependently reduced in 8-G-treated platelets. Filamin A is the linker between GPIbα and the membrane skeleton. Considering the results of associations of GPIbα with the cytoskeleton in response to different gravity conditions (8, 11), these data suggest that the association of filamin A with the actin cytoskeleton, other than the association of filamin A with GPIbα, is regulated by gravity.

Effects of simulated microgravity and hypergravity on actin cytoskeleton organization. The actin cytoskeleton is very sensitive to changes of gravity, and remodulation of the actin cytoskeleton and variations of the total tk;2amounts of actin in response to gravity changes have been reported to occur in many kinds of cells (18, 30). To further investigate the effects of different gravities on platelet cytoskeleton, washed human platelets stimulated by modeled microgravity or hypergravity at 8 G were stained with rhodamine-phalloidin specific for F-actin. Control platelets kept in 1-G condition showed only a small amount of actin fibers (Fig. 2A). Compared with 1-G controls, the actin filaments were reduced and gathered in a ring around the plasma membrane layer in platelets treated with modeled microgravity (Fig. 2A). In contrast, more actin fibers were presented and distributed in the cytosol of platelets stimulated by hypergravity at 8 G (Fig. 2B). Together, these results indicate that hypergravity induces actin filament formation and redistribution, whereas actin filaments are reduced in platelets under microgravity conditions.

Hypergravity induces intracellular Ca2+ release in human platelets. Changes of [Ca2+]i in altered gravity conditions have been reported to occur widely in many types of cells and to associate with cytoskeletal reorganization (16, 17). [Ca2+]i plays key roles in regulating platelet functions (21). Thus we examined [Ca2+]i levels in platelets stimulated by hypergravity. As shown in Fig. 3A and B, in contrast to the 1-G control, hypergravity induced a significant increase in [Ca2+]i in platelets. In addition, we also examined [Ca2+]i levels in platelets stimulated with modeled microgravity. However, no detectable difference...
in [Ca^{2+}], between platelets treated with modeled microgravity and 1-G controls was obtained (data not shown).

The elevation of [Ca^{2+}] is dependent on either extracellular Ca^{2+} entry or intracellular Ca^{2+} release, or both (15). To determine whether the elevation of [Ca^{2+}] results from the extracellular Ca^{2+} entry in platelets responding to hypergravity stimulus, we treated fluo 3-loaded platelets with the Ca^{2+} chelator EDTA (or EGTA) before application of hypergravity. As shown in Fig. 3, A and B, addition of EDTA did not significantly reduce the [Ca^{2+}] levels in platelets treated with hypergravity. Furthermore, the chelation of extracellular Ca^{2+} had no effect on [Ca^{2+}] levels in 1-G platelets (data not shown). Together, these data indicate that hypergravity selectively elicits Ca^{2+} release from intracellular organelle(s), rather than promoting Ca^{2+} influx via mechanosensitive Ca^{2+} channels in the plasma membrane.

Role of [Ca^{2+}] in ristocetin- and ADP-induced platelet aggregation. The elevation of [Ca^{2+}] is sufficient for promoting cytoskeletal reorganization (25); on the other hand, reorganization of the actin cytoskeleton also modulates intracellular Ca^{2+} release (4, 35). To investigate the association of [Ca^{2+}] with cytoskeletal reorganization in platelets under hypergravity conditions, we pre-treated washed human platelets with BAPTA-AM, a membrane-permeable Ca^{2+} chelator, for 20 min before application of 8-G hypergravity. As shown in Fig. 4A, chelation of intracellular Ca^{2+} by BAPTA-AM did not attenuate the hypergravity-induced formation and distribution of actin filaments in the cytosol of platelets. These data indicate that the change in actin cytoskeleton is upstream or independent of intracellular Ca^{2+} release in platelets exposed to hypergravity.

Ristocetin-induced platelet aggregation was enhanced in hypergravity-treated platelets (8, 11). To investigate the role of

Fig. 2. Effects of simulated MG and hypergravity on platelet actin cytoskeleton. Washed human platelets were exposed to simulated MG or 1 G for 1 h (A) or to 8-G hypergravity or 1 G for 15 min (B) and then fixed with 4% paraformaldehyde and stained using rhodamine-conjugated phalloidin. Note that 1-G control platelets contained few actin filaments in cytosol, whereas the actin fibers were converged into peripheral ruffles after the application of simulated MG for 1 h. In contrast, dense actin filaments were formed in cytosol after the application of 8-G hypergravity for 15 min. Representative results of 3 independent experiments are shown.

Fig. 3. Hypergravity induces intraplatelet Ca^{2+} release. A: washed human platelets were loaded with fluo-3 AM and then exposed to 8 or 1 G for 15 min in the presence or absence of 5 mM EDTA (or EGTA). The intracellular Ca^{2+} level ([Ca^{2+}]) was analyzed using flow cytometry. Representative histograms of fluo-3 fluorescence of platelets are shown. B: the geometric mean fluorescence intensity of fluo-3 AM binding is indicated. Data are means ± SD of 3 independent experiments. **P < 0.01 compared with 1-G control.
[Ca\textsuperscript{2+}], in ristocetin-induced platelet aggregation, PRP was pretreated with BAPTA-AM before hypergravity at 8 G and then induced to aggregate with ristocetin. The low concentration of ristocetin was used to induce a relatively lower aggregation in 1-G control platelets that would sensitively reflect platelet functional variations. Compared with the synchronous 1-G controls, ristocetin-induced platelet aggregation was significantly increased after exposure of platelets to hypergravity, whereas BAPTA-AM significantly reduced ristocetin-induced aggregation of hypergravity-exposed platelets (Fig. 4, B and C); in contrast, BAPTA-AM had no effect on the aggregation of ristocetin-induced control platelets (data not shown).

In addition, our prior findings suggest that the platelet signaling system and reaction tend to be exhausted in hypergravity conditions, and thus hypergravity-exposed platelets exhibit decreased response to ADP (8, 11). To further explore the effect of intracellular Ca\textsuperscript{2+} release on platelet signaling system, we tested ADP-induced platelet aggregation. PRP was pretreated with BAPTA-AM before hypergravity and then induced to aggregate with ADP. Consistent with our previous data, 8 G-exposed platelets displayed a decreased response to ADP compared with 1-G controls (Fig. 4, D and E). However, BAPTA-AM obviously enhanced ADP-induced platelet aggregation (Fig. 4, D and E), indicating that the platelet signaling system and reaction are relatively more intact in BAPTA-AM/ hypergravity-treated platelets than in hypergravity-treated platelets. Compared with hypergravity-treated platelets, BAPTA-AM did not affect ADP-induced control platelet aggregation (data not shown). Together, these results indicate that intracellular Ca\textsuperscript{2+} release incurred by hypergravity is not required for actin cytoskeleton reorganization of platelets but is involved in the regulation of platelet function. The cytoskeletal reorganization appears to be upstream or independent of intracellular Ca\textsuperscript{2+} release in response to hypergravity stimulation.

**Effects of aspirin on in vivo hemostasis in mice under hypergravity condition.** Our previous studies have demonstrated that hypergravity-treated platelets are hypersensitive to agonists (8, 11, 19). Particularly, the tail bleeding time was significantly shortened in mice exposed to high-G force, and platelet thrombi were disseminated in the heart and circulation system from the mice that died from high-G force (8, 11). Therefore, to further confirm the role of hypergravity in platelet activation in vivo and to find the approaches of preventing or curing hypergravity-related thrombotic diseases, we examined the effect of aspirin on in vivo hemostasis in mice under hypergravity conditions. Consistent with our previous findings (8, 11), the median tail bleeding time was significantly shortened in mice exposed to hypergravity at 12 G in contrast to that
of synchronous 1-G controls, and pathological analysis revealed that platelet thrombi disseminated in the heart ventricle and blood vessels all over the brain, lung, and heart from the mice that died from 12-G treatment (data not shown). However, the shortened tail bleeding time was obviously reversed by aspirin (Fig. 5A). We also calculated the death rate of mice treated with hypergravity. As shown in Fig. 5B, aspirin significantly reduced the death rate of hypergravity-treated mice. Furthermore, aspirin treatment did not cause increased spontaneous bleeding in 1-G control mice, supporting the rationality of the dose used. In addition, the rebleeding rate of aspirin/hypergravity-treated mice was higher than that of controls (Fig. 5C), indicating the inhibition of platelet function. Together, these data suggest that aspirin effectively inhibits platelet activation induced by hypergravity in mice.

**Effects of tirofiban on in vivo hemostasis in mice under hypergravity condition.** During platelet thrombus formation, the stable platelet adhesion and aggregation depend on integrin αIIbβ3 activation (17). Thus another anti-platelet agent, tirofiban, was used to further explore the countermeasure against platelet hyperactivity under hypergravity. Similar to the data obtained by aspirin, the median tail bleeding time of tirofiban/hypergravity-treated mice was almost comparable to that of synchronous 1-G controls but was significantly longer than that of hypergravity-treated mice (Fig. 6A). The death rate was significantly reduced in tirofiban/hypergravity-treated mice compared with hypergravity-treated mice (Fig. 6B). Furthermore, the rebleeding rate of tirofiban/hypergravity-treated mice and 1-G controls was significantly higher than that in hypergravity-treated mice (Fig. 6C). Thus the data suggest that the anti-platelet agent tirofiban potently inhibits platelet-related thrombus formation in mice under hypergravity conditions.

Aspirin administration effectively inhibits platelet activation in mice. P-selectin is a transmembrane protein normally presents in the intracellular α-granules of platelets, and it is rapidly translocated to the platelet surface upon platelet activation (29). Thus P-selectin is known as a well-recognized surface marker of platelet activation. To further confirm whether aspirin inhibits hypergravity-induced platelet activation in vivo, we assessed the surface expression of P-selectin in platelets from hypergravity-treated mice by using flow cytometry. There was a significant increase in P-selectin surface level in platelets from hypergravity-treated mice compared with those from 1-G controls (Fig. 7A). However, the increased P-selectin expression was obviously reversed in platelets from mice treated with aspirin and hypergravity (Fig. 7, A and B). These data further indicate that anti-platelet agent specifically inhibits platelet activation induced by hypergravity in vivo.

**DISCUSSION**

We reported recently that the platelet functions are inhibited in microgravity environments and activated under high-G conditions. However, the mechanisms of platelet functional alterations remain unclear. The data described in this study demonstrate that association of GPIbα with filamin A and the organization of actin cytoskeleton are regulated by gravity. Intracellular Ca2+ levels were elevated by hypergravity, which upregulated ristocetin-induced platelet aggregation. Anti-platelet agents effectively reversed activation of platelets induced by hypergravity and reduced the death rate of mice exposed to hypergravity.

Filamin A, which directly associates with the actin cytoskeleton, anchors the GPIb-IX complex to the membrane skeleton

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**Fig. 5. Tail bleeding times in 1-G control, hypergravity-treated, or aspirin/hypergravity-treated mice.** The 4- to 6-wk-old C57 black mice were anesthetized with an intraperitoneal injection of pentobarbital sodium. The anesthetized mice were intraperitoneally injected with aspirin or alcohol and then subjected to hypergravity at 12 or 1 G for 15 min. Tails of differently treated mice were transected 0.5 mm from the end and immersed in 0.15 M NaCl maintained at 37°C. The time when bleeding was arrested was noted as the bleeding time. A: symbols indicate the bleeding times of individual mice; bars represent the median bleeding time of each group. Note that aspirin treatment effectively prolonged tail bleeding times compared with hypergravity-treated mice and did not cause spontaneous bleeding in 1-G control mice. B: percentage of mice that died during different treatments. Results were calculated from measurements in a total of 72 mice. C: percentage of mice that showed rebleeding. Results were calculated from measurements in a total of 72 mice.
In the present study, the amount of filamin A coimmunoprecipitated with GPIb-H9251 was enhanced in the soluble components of platelets treated with modeled microgravity and, in contrast, was reduced in 8 G-treated platelets. In our previous findings, GPIb-H9251 surface expression and association with the cytoskeleton were significantly decreased in platelets exposed to simulated microgravity and obviously increased in hypergravity-exposed platelets (8, 11). Combined with our prior results, these data suggest that the association of filamin A with cytoskeleton, other than the association of filamin A with GPIb-H9251, was regulated by gravity. Furthermore, consistent with other cell types (2, 16, 24), hypergravity induced actin filament formation and reorganization in platelets, whereas actin filaments were reduced in platelets under microgravity conditions in the current observations. Thus these data suggest that hypergravity induces actin filament formation and the interaction of filamin A with cytoskeleton, which results in the increase of GPIb-IX surface expression; in contrast, interactions of filamin A with cytoskeleton and GPIb-IX surface expression are reduced in platelets under modeled microgravity conditions.

Elevation of [Ca\(^{2+}\)]\(_i\) incurs cytoskeletal reorganization (3, 33); on the other hand, the dynamic changes in the actin cytoskeleton also induce intracellular Ca\(^{2+}\) release (4). In the current study, we found that [Ca\(^{2+}\)]\(_i\) was elevated in platelets in response to hypergravity but not to simulated microgravity. The increase of [Ca\(^{2+}\)]\(_i\) is dependent on either extracellular Ca\(^{2+}\) entry or the release of Ca\(^{2+}\) from intracellular stores, or both. Although many reports indicate the sensitivity of membrane-bound Ca\(^{2+}\) channels in many cell type responses to mechanical stimuli (3), we have demonstrated that removal of extracellular Ca\(^{2+}\) with the chelator EDTA (or EGTA) did not reduce the elevation of [Ca\(^{2+}\)]\(_i\) in response to hypergravity in platelets, excluding the involvement of Ca\(^{2+}\) channels. To determine the interaction between release of intracellular Ca\(^{2+}\) and cytoskeletal remodeling under hypergravity conditions, we...
examined the effect of the intracellular Ca\(^{2+}\) chelator BAPTA-AM on changes of actin cytoskeleton, and the data indicate that addition of BAPTA-AM did not prevent the changes in cytoskeletal reorganization, excluding the necessity of intracellular Ca\(^{2+}\) release for actin reorganization. However, whether Ca\(^{2+}\) release in response to hypergravity is a downstream event or is independent of actin reorganization remains to be further investigated.

In platelets, elevation of [Ca\(^{2+}\)]\(_i\) is involved in the regulation of almost every important function of platelets such as adhesion, secretion, and aggregation (13). Furthermore, elevation of [Ca\(^{2+}\)]\(_i\) leads to platelet hyperactivity (20). Thus we investigated the role of [Ca\(^{2+}\)]\(_i\) in platelet hyperactivity induced by hypergravity. The data indicate that [Ca\(^{2+}\)]\(_i\) increase plays a key role in platelet hyperactivity under hypergravity conditions. An increase of GPIb-IX surface expression by itself cannot incur platelet activation. Thus the elevation of [Ca\(^{2+}\)]\(_i\), might present as the main reason leading to platelet hyperactivity under hypergravity conditions.

Aspirin is widely used as an antithrombotic drug that effectively blocks the arachidonate pathway by inhibiting prosta-glandin synthesis, thus preventing platelet activation and reducing the rate of thrombotic formation (23). Tirofiban is a novel nonpeptide platelet antagonist and has a high degree of selectivity for the platelet \(\alpha I\beta 3\) receptor (6). Therefore, to further confirm the role of hypergravity in platelet activation in vivo and to preliminarily explore the countermeasure against hypergravity-related thrombotic diseases, we examined the effects of aspirin and tirofiban on in vivo hemostasis in mice under hypergravity conditions. The data indicate that administration of aspirin or tirofiban effectively inhibited in vivo platelet activation induced by hypergravity and reduced death rate in mice under hypergravity conditions, which not only further confirms the activation of platelets in vivo but also suggests the possibility of preventing thrombus formation in a hypergravitational environment.

In conclusion, the association of GPIb\(_o\) with filamin A and the organization of actin cytoskeleton were regulated by diff-erent gravity conditions. Intracellular Ca\(^{2+}\) levels were ele-vated by hypergravity, which obviously enhanced ristocetin-induced platelet aggregation. Two anti-platelet agents, aspirin and tirofiban, effectively reversed activation of platelets in-duced by hypergravity in vivo and reduced the death rate of mice exposed to hypergravity. Thus these data implicate the mechanisms underlaying the functional variations of platelets under different gravity conditions and also suggest an effective countermeasure against hypergravity-related thrombotic dis-eases.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


