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ORIGINAL ARTICLE

Hyperthermia induces platelet apoptosis and glycoprotein Ibα ectodomain shedding

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Abstract

Hemorrhage is a significant pathological feature of some fever or hyperthermia-related diseases, such as dengue fever and heatstroke. Although the mechanisms of hemorrhage in these diseases are thought to be complex, whether there is an association between hemorrhage and hyperthermia or fever remains unclear. Platelets play a central role in maintaining integrity of endothelium and biological hemostasis. To explore the effect of hyperthermia on platelet physiology, platelet-rich plasma or washed platelets were incubated at hyperthermia (22°C), normothermia (37°C) or hyperthermia (40 and 42°C) for 1 or 2 hours. ADP and α-thrombin induced platelet aggregations were obviously reduced in platelets incubated at hyperthermia. Hyperthermia induced apoptotic events in platelets, including depolarization of mitochondrial inner transmembrane potential, caspase-3 dependent gelsolin cleavage and phosphatidylserine exposure. Furthermore, hyperthermia incurred platelet glycoprotein Ibα ectodomain shedding. Thus, these data suggest that hyperthermia induces platelet apoptosis and dysfunction. These findings have important implications for the pathogenesis of hemorrhage in fever or hyperthermia-related diseases, and also suggest that attention should be paid to platelet apoptosis under relatively high temperature conditions.

Keywords: Platelets, apoptosis, hemorrhage, hyperthermia, fever, GPIba shedding

Introduction

In some patients with fever or hyperthermia, hemorrhage is a significant pathological feature, which incurs severe or even fatal consequence. Heatstroke, which led to an unprecedented number of deaths in Europe in the summer of 2003, is characterized by a core temperature rising rapidly to above 40°C [1, 2]. Postmortem findings in patients that died from heatstroke include diffuse bleeding, hemorrhagic necrosis and widespread microthrombi in most organs of the body [3]. Fever and hemorrhage have emerged as typical symptoms for many viral-related diseases [4–7] such as dengue fever, endemic in many parts of Asia and America with an estimated 100 million cases each year [4, 5], hantavirus pulmonary syndrome [6] and a viral hemorrhagic syndrome induced by an African swine fever (ASF) virus [7]. In addition, thrombocytopenia and hemorrhagic risk also occur in patients with malignant diseases, particularly in patients undergoing hyperthermia therapy [8]. Although the mechanisms of hemorrhage in patients with hyperthermia or fever have been thought to be complex, whether there is an association between hemorrhage and hyperthermia or fever is not well understood.

Platelets play a central role in maintaining integrity of endothelium and biological hemostasis. Upon endothelium injury, the interaction of platelet glycoprotein (GP) Ibα with von Willebrand factor initiates platelet adhesion and simultaneously triggers intracellular signaling cascades leading to platelet aggregation to stop bleeding [9]. Thus, many efforts have been made to investigate the role of platelets in fever or hyperthermia-related hemorrhagic diseases. Platelet count obviously reduces in most patients with dengue fever [4].

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2  Z. Wang et al.

or heatstroke [1]. And reduced platelet aggregations
have been found in patients with hemorrhage fever
[5], raising the possibility that fever or hyperthermia
incurs reduction of platelet count or function leading
to hemorrhage. Fever or hyperthermia induces a
series of physiological responses including cell
apoptosis in human body [1, 7]. There is also a
report indicating that apoptotic markers are
increased in platelets stored at a relatively high
temperature [10], suggesting the possible association
of temperature with platelet apoptosis.

Platelet apoptosis induced by physiological [11]
or chemical [12–15] compounds, or platelet storage
[16, 17] has been reported to occur widely in vivo
and in vitro. Up to now, most of the platelet
apoptotic events appear to arise from the mitochon-
drial pathway, characterized by depolarization
of mitochondrial inner transmembrane potential
(DΨm, caspase-3 activation and verifications of the
Bcl-2 family protein expression [11, 17, 18].

Furthermore, phosphatidylserine (PS) exposure
also occurred in platelets stimulated with strong
agonists such as A23187 [15], thrombin [11]
or platelet storage under blood banking conditions
[16, 17]. PS exposure occurs in both platelet
activation and apoptosis. However, the signaling
pathways leading to PS exposure are distinct between
the two processes [19], suggesting that platelet
activation and apoptosis occur separately under
physiological conditions. Platelet apoptosis reported
to be induced by natural platelet agonist thrombin or
pathological high shear stress, leads to the dysfunc-
tion and clearance of circulatory platelets. Thus,
to investigate whether there is an association between
platelet apoptosis and hyperthermia or fever has
important implications for the pathogenesis of hem-
orrhage in fever or hyperthermia related diseases.

In this study, we demonstrate that physiological
agonists-induced platelet aggregations were gradually
reduced in platelets treated with increasing tem-
peratures. Hyperthermia induces apoptotic events
in human platelets, including DΨm reduction,
caspase-3 dependent gelsolin cleavage and PS
externalization. Furthermore, hyperthermia incurs
platelet GPIIb or ectodomain shedding.

Materials and methods

Reagents

Monoclonal antibody SZ2 against GPIIb or was kindly
provided by Dr. Changgeng Ruan (Soochow
University, Suzhou, China). Anti-gelsolin monoclo-
nal antibody, α-thrombin and ADP were purchased
from Sigma (St. Louis, MO, USA), FITC-conju-
gated goat anti-mouse IgG and HRP-conju-
gated goat anti-mouse IgG were purchased from
Santa Cruz Biotechnology (Santa Cruz, CA, USA).
Caspase-3 inhibitor z-DEVD-fmk, FITC-conjugated
annexin V and tetramethylrhodamine ethyl ester
(TMRE) were purchased from Bender Medsystem
(Vienna, Austria). Calpain inhibitor carbenoxo-
valin-phenylalanilinal (MDL 28170) was purchased
from Calbiochem (San Diego, CA, USA).

Preparation of washed platelets

Blood from healthy volunteers was anti-
coagulated with 1/9 volume of 3.8% trisodium citrate
for platelet-rich plasma (PRP) or 1/7 volume of acid-
citratedextrose (ACD, 2.5% trisodium citrate, 2.0%
D-glucose, 1.5% citric acid) for washed platelets.

After centrifugation, PRP was isolated. For washed
platelets, PRP was centrifugated. Isolated platelets
were washed twice with CGS buffer and re-suspended in modified Tyrode’s buffer (2.5 mM
Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM
NaHCO3, 5.5 mM D-glucose, pH 7.4) to a final
density of 108/ml, and then incubated at room temperature (RT) for 1 hour (h) to recover
to resting state as described previously [20, 21].

Platelet aggregation assay

For platelet aggregation studies, 0.3 ml of PRP
or washed platelets were incubated at different
temperatures (RT, 37°C, 40°C or 42°C) for 1
or 2h, and then were immediately added to glass
aggregometer cuvettes. Platelet aggregation was
induced by the addition of α-thrombin or ADP and
monitored by a turbidimetric platelet aggregometer
(Xinpusen, Beijing, China) at 37°C with a stirring
speed at 1000 rpm.

ΔΨm measurement

Washed platelets (5 × 107/ml) in modified Tyrode’s
buffer were incubated at RT, 37°C, 40°C, or 42°C
for 2h. ΔΨm was determined using the potential
sensitive dye tetramethyl-rhodamine-ethylen-
ether (TMRE). Briefly, TMRE was added into the treated
platelets in a 100 nM final concentration. Then
samples were further incubated in the dark at 37°C
for 2h, and then were immediately added to glass
and analysed by flow cytometry. TMRE signals were excited using a 488 nm krypton-
argon laser line and its emissions were captured
using filters at 625 nm.

Gelsolin cleavage assay

Washed platelets (3.0 × 108/ml) in modified
Tyrode’s buffer were incubated at RT, 37°C, 40°C
or 42°C for 2h and lysed with an equal volume of
lysis buffer containing 0.1 mM E64, 1 mM phenyl-
methylsulfonyl fluoride (PMSF) and 1/100 aprotinin
on ice for 30 min. Whole lysate was added with
one-fourth volume of five times sodium dodecyl
sulfate (SDS) sample buffer, resolved by SDS-PAGE
and immunoblotted with anti-gelsolin antibody (1:2500). In inhibition experiments, platelets were pre-incubated with calpain inhibitor MDL 28170 (100 μM), caspase-3 inhibitor z-DEVD-fmk (50 μM) or vehicle (DMSO) at RT for 15 min. Platelets were then incubated at different temperatures for 2 h. The final concentration of DMSO was 0.5%.

**PS externalization assay**

Washed platelets (5 × 10^7/ml) in modified Tyrode's buffer were incubated at RT, 37°C, 40°C, or 42°C for 2 h. Annexin V binding buffer was mixed with pre-treated platelets and annexin V-FITC at a 50:10:1 ratio. Samples were gently mixed by rocking and incubated at RT for 15 min in the dark, then analysed by flow cytometry. In inhibition experiments, platelets were pre-incubated with caspase-3 inhibitor z-DEVD-fmk (50 μM) or vehicle (DMSO) at RT for 15 min before treating platelets for 2 h at different temperatures.

**Flow cytometric analysis of GPIbα ectodomain shedding**

Washed platelets (5 × 10^7/ml) in modified Tyrode’s buffer were incubated at RT, 37°C, 40°C or 42°C for 2 h. Platelet suspension (50 μl) was incubated with SZ2 or mouse IgG (5 μg/ml) as negative control at RT for 30 min. After washing, platelets were further incubated with FITC-labeled Goat-anti-Mouse IgG antibody in the dark at RT for 30 min, then analysed by flow cytometry.

**Western blot analysis of GPIbα ectodomain shedding**

Washed platelets (3.0 × 10^8/ml) in modified Tyrode’s buffer were incubated at RT, 37°C, 40°C or 42°C for 2 h. Samples were centrifuged at 4000 rpm for 5 min to harvest the supernatants. Each supernatant was added with one-fourth volume of 5 × SDS sample buffer, resolved by SDS-PAGE and immunoblotted with the anti-GPIbα N-terminal antibody SZ2. In inhibition experiments, platelets were pre-incubated with calpain inhibitor MDL 28170 (100 μM), caspase-3 inhibitor z-DEVD-fmk (50 μM) or vehicle (DMSO) at RT for 15 min. Platelets were then incubated at different temperatures for 2 h.

**Statistical analysis**

Data are shown as means ± SD. The statistical difference between two groups was determined by the paired Student’s t-test. A p-value less than 0.05 was considered significant. Bonferroni correction was applied to adjust for multiple comparisons.

**Results**

**ADP and α-thrombin induced platelet aggregations are gradually reduced with the increase of temperature and time duration**

Previous reports have shown that agonist-induced platelet aggregations are reduced in platelets pre-treated with heat in vitro or in platelets from patients with fever and heatstroke [5, 22]. To explore the effect of hyperthermia on platelet function, PRP was isolated and incubated at hypothermia (22°C), normothermia (37°C) or hyperthermia (40 or 42°C) for 1 or 2 h, and then induced to aggregation by ADP. Platelet aggregation was apparently reduced with the growth of temperature in a time-dependent manner (Figure 1A, B). To exclude the possible interference from plasma proteins and to further investigate the effects of hyperthermia on platelet function, α-thrombin induced platelet aggregations were examined in washed platelets incubated at different temperatures for 1 or 2 h. Washed platelets presented normal aggregation response to α-thrombin in platelets incubated at hypothermia, whereas α-thrombin induced platelet aggregation reduced gradually with the increase of temperature and time duration (Figure 1C, D). Taken together, these data indicate that physiological agonist-induced platelet aggregations reduce with an increase of temperature and time duration.

**Hyperthermia induces depolarization of ΔΨ_m in platelets**

Fever or hyperthermia elicits cell apoptosis in the human body [1, 7]. Furthermore, it was reported that apoptotic markers were increased in platelets stored at 37°C [10], suggesting the possibility that platelets could be induced to apoptosis by hyperthermia. Thus, we investigated whether reduced platelet aggregations resulted from platelet apoptosis under high temperature conditions. There are two distinct pathways, the TNF family death receptor pathway and the mitochondrial pathway, leading to apoptosis. Therefore, the effect of hyperthermia on platelet ΔΨ_m depolarization was analysed by cell-permeable lipophilic cationic dye TMRE, which is accumulated in the mitochondrial matrix, driven by depolarization of ΔΨ_m [29]. As shown in Figure 2, ΔΨ_m depolarization rates were enhanced in platelets incubated at 37 or 40°C particularly, and were significantly enhanced in platelets incubated at 42°C.
This suggests apoptotic events are induced by hyperthermia in platelets.

Hyperthermia induces gelsolin cleavage in platelets

Caspase-3 is one of executioners in the caspase family, which, once activated, cleaves a variety of substrates leading to the dismantling and collapse of the scaffolding of the cell [10–14]. Gelsolin is a cytoskeletal regulatory protein that has been shown to be a specific substrate of caspase-3 [26]. Therefore, to further confirm mitochondria-dependent platelet apoptosis induced by hyperthermia, caspase-3 activation was measured by detecting the gelsolin cleavage in platelets. Gelsolin cleavage was not detected in platelets incubated at RT or 37°C for 2 h, while it obviously occurred in platelets incubated at hyperthermia (Figure 3A). Thus these data further suggest hyperthermia induces platelet apoptosis.

Although it is widely accepted that gelsolin is a caspase-3 specific substrate, there is also a report suggesting that gelsolin could be cleaved by calpain [15]. In order to investigate whether calpain was involved in hyperthermia induced gelsolin cleavage, platelets were pre-incubated with calpain inhibitor with caspase-3 inhibitor as positive control. The results showed that gelsolin cleavage was inhibited by the caspase-3 inhibitor z-DEVD-fmk (Figure 3B), whereas it was not inhibited by calpain inhibitor MDL 28170 (Figure 3C), indicating that hyperthermia-induced gelsolin cleavage is mediated by caspase-3 but not calpain. We also examined calpain activity in hyperthermia treated platelets by detecting talin cleavage, another substrate of calpain [27]. No talin cleavage was detected under the current conditions (data not shown), indicating that calpain was not activated in hyperthermia treated platelets. Taken together, these data indicate that hyperthermia induces caspase-3 dependent gelsolin cleavage in platelets.

Hyperthermia induces platelet PS exposure

Both weak and strong platelet agonists induce depolarization of ΔΨm and caspase-3 activation
However, only strong agonists could induce PS exposure during the platelet apoptotic process [11, 24]. To further investigate whether hyperthermia is strong enough to induce PS exposure during platelet apoptosis, PS exposure was examined in platelets incubated at different temperatures by measuring annexin V binding. Consistent with other apoptotic markers, PS exposure was temperature-dependently presented in platelets incubated at RT, 37°C, 40°C or 42°C for 2 h (Figure 4A, B).

PS exposure is thought to be a typical event in both platelet activation and apoptosis; however, the signaling pathways leading to PS exposure are quite different between these two processes [19]. To clarify that PS exposure resulted from platelets apoptosis in the current observations, caspase-3 inhibitor z-DEVD-fmk was pre-incubated with platelets before hyperthermia treatment. Figure 4C shows that inhibition of caspase-3 significantly reduced PS exposure in platelets incubated at 40°C or 42°C, suggesting that PS exposure is resulted from platelet apoptosis. These data indicate that hyperthermia presents as a strong platelet agonist eliciting platelet apoptotic events including PS exposure.

Hyperthermia induces platelet GPIbα ectodomain shedding

Physiological or chemical compounds such as thrombin and A23187 or high shear stress induces platelet apoptosis and also could induce platelet GPIbα ectodomain shedding [11, 24, 28]. GPIbα is the main platelet adhesion receptor playing a key role in initiating platelet adhesion and thrombus formation. GPIbα ectodomain shedding is a physiological regulatory mechanism leading to platelet dysfunction [29]. In order to investigate whether GPIbα ectodomain shedding is involved in hyperthermia-related platelet dysfunction, GPIbα shedding was examined in platelets incubated at hyperthermia. GPIbα surface expression was significantly reduced with the...
increase of temperature (Figure 5A, B), indicating that GPIbα extracellular peptides were cleaved from the surface of platelets. Furthermore, the N-terminal GPIbα peptides were gradually presented in the supernatants of platelets with the increase of temperature (Figure 5C).

To further investigate whether GPIbα shedding was the result of hyperthermia induced platelet apoptosis, caspase-3 inhibitor z-DEVD-fmk was pre-incubated with platelets. Hyperthermia-induced GPIbα shedding was not inhibited by caspase-3 inhibitor (data not shown), indicating that hyperthermia-induced GPIbα shedding is upstream or unrelated with caspase-3 activation. In addition, calpain inhibitor MDL 28170 also did not inhibit hyperthermia-induced platelet GPIbα ectodomain shedding (data not shown).

Discussion

The results presented here demonstrate that hyperthermia induces apoptotic events in human platelets, including ΔΨm reduction, caspase-3 dependent gelsolin cleavage and PS externalization. Physiological agonist-induced platelet aggregations gradually reduce in platelets incubated at increasing temperatures. Furthermore, hyperthermia incurs platelet GPIbα ectodomain shedding.

Platelets play a central role in maintaining the integrity of endothelium and biological hemostasis. In order to explore the possible association of hemorrhage with fever or hyperthermia, we first investigated the effect of hyperthermia on platelet function. The results indicated that platelet aggregations were reduced in platelets incubated at hyperthermia. Next, we tried to explore the mechanism underlying the reduced platelet aggregation under high temperature conditions. It has been reported that fever or hyperthermia resulted in cell apoptosis in human body [1, 7] and apoptotic markers were increased in platelets stored at a relatively high temperature [10], suggesting the possibility that platelets could be induced to apoptosis by hyperthermia. Thus, platelet apoptotic markers were examined in platelets incubated at different temperatures and the data indicated that platelet apoptosis is induced by hyperthermia.

Platelet apoptosis occurs widely in platelets stimulated with physiological or chemical compounds, shear stress or in stored platelets [11–17, 24]. In nucleated cells, it is well documented that two main distinct pathways lead to apoptosis: the extrinsic pathway initiated via the cell surface death receptors; and the intrinsic pathway triggered by changes in mitochondrial integrity, including depolarization of ΔΨm and release of apoptotic cofactors from the mitochondrial intermembrane space [11, 30]. Up to now, most of the platelet apoptosis events appear to arise from the mitochondrial pathway [11, 16, 24]. In the current observation, hyperthermia induces ΔΨm reduction and gelsolin cleavage in platelets, indicating that hyperthermia-induced platelet apoptosis is also elicited via the intrinsic pathway. In addition, hyperthermia is reported to engage in the intrinsic mitochondrial pathway rather than the extrinsic death receptor pathway in nucleated cells [31], thus...
supporting our findings. Hyperthermia-induced apoptosis in nucleated cells has been thought to occur via the damage of different cellular components such as membrane, chromatin, nuclear matrix structure and DNA [32]. Whether the same signaling cascades also occur in platelets is still unclear.

Although gelsolin is thought to be a caspase-3 specific substrate cleaved during apoptosis, there is also a report suggesting that gelsolin could be cleaved by calpain [15]. Furthermore, PS exposure is a typical event in both platelet activation and apoptosis; however, the signaling pathways leading to PS exposure are quite different between these two processes [19]. Thus, the causality of gelsolin cleavage and PS exposure as being due to apoptosis was established by examining the effects of caspase-3 and calpain inhibitors. The data indicated that inhibition of caspase-3 potently inhibited hyperthermia-induced gelsolin cleavage and PS exposure. However, calpain was not activated in hyperthermia-exposed platelets as detected by talin cleavage, thus calpain inhibitor had no effect on hyperthermia-induced platelet apoptotic events. Therefore, these data indicate that gelsolin cleavage and PS exposure are special apoptosis events in hyperthermia treated platelets.

In addition, we found that GPIbα, the main platelet adhesion receptor, was shed from platelets under high temperature conditions. This finding further deciphers the reason of platelet dysfunction at hyperthermia. However, whether there is an association between GPIbα ectodomain shedding and platelet apoptosis is still unknown. In the current observation, inhibition of caspase-3 has no effect on hyperthermia-induced GPIbα shedding, suggesting that hyperthermia induced GPIbα shedding through a caspase-3 independent pathway. In addition, our previous report showed that inhibition of calpain led to the elimination of GPIbα shedding [33], therefore indicating that calpain plays a key role in GPIbα ectodomain shedding. However, calpain was not activated in hyperthermia-exposed platelets, an calpain inhibitor had no effect on hyperthermia-induced GPIbα shedding (data not shown). Thus the mechanism underlying hyperthermia induced GPIbα shedding needs further investigation.

Hemorrhage is a significant pathological feature in some fever or hyperthermia-related diseases such as dengue fever and heatstroke, which are two severe
diseases in terms of human morbidity and mortality. Although the mechanisms of hemorrhage have been explored in hyperthermia or fever-related diseases, up to now little is known about the linkage between hemorrhage and the body temperature. In this study, the data demonstrate that hyperthermia induces platelet apoptosis, which clarifies that platelets treated with hyperthermia have impaired platelet function and changed morphology [22], and decreased platelet count or function in some fever or hyperthermia-related diseases [1, 4, 8]. These findings not only have important implications for the pathogenesis of hemorrhage in some fever or hyperthermia-related diseases, but also suggest that attention should be paid to platelet apoptosis under relatively high temperature conditions, such as during hyperthermia therapy or platelet storage.

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References

14. Z. Wang et al.


