Platelet Dense-Granule Secretion Plays a Critical Role in Thrombosis and Subsequent Vascular Remodeling in Atherosclerotic Mice

Sarah M. King, PhD; Rachel A. McNamee, BS; Aiilyan K. Houng, BS; Rakesh Patel, MD; Michael Brands, PhD; Guy L. Reed, MS, MD

Background—Platelet aggregation plays a critical role in myocardial infarction and stroke; however, the role of platelet secretion in atherosclerotic vascular disease is poorly understood. Therefore, we examined the hypothesis that platelet dense-granule secretion modulates thrombosis, inflammation, and atherosclerotic vascular remodeling after injury.

Methods and Results—Functional deletion of the Hermansky-Pudlak syndrome 3 gene (HPS3−/−) markedly reduces platelet dense-granule secretion. HPS3−/− mice have normal platelet counts, platelet morphology, and α-granule number, as well as maximal secretion of the α-granule marker P-selectin; however, their capacity to form platelet-leukocyte aggregates is significantly reduced (P<0.05). To examine the role of platelet dense-granule secretion in these processes, atherosclerosis-prone mice with combined genetic deficiency of apolipoprotein E and HPS3 (ApoE−/−, HPS3−/−) were compared with congenic, atherosclerosis-prone mice with normal platelet secretion (ApoE−/−, HPS3+/+). After 16 to 18 weeks on a high-fat diet, both groups of mice had similar fasting cholesterol levels and body weight. Carotid arteries of ApoE−/−, HPS3+/+ mice thrombosed rapidly after FeCl3 injury, but ApoE−/−, HPS3−/− mice were completely resistant to thrombotic arterial occlusion (P<0.01). Three weeks after injury, neointimal hyperplasia (from α-smooth muscle actin–positive cells) was significantly less (P<0.001) in arteries from ApoE−/−, HPS3−/− mice. In ApoE−/−, HPS3−/− mice, there were also pronounced reductions in arterial inflammation, as indicated by a 74% decrease in CD45-positive leukocytes (P<0.01) and a 73% decrease in Mac-3–positive macrophages (P<0.05).

Conclusions—In atherosclerotic mice, reduced platelet dense-granule secretion is associated with marked protection against the development of arterial thrombosis, inflammation, and neointimal hyperplasia after vascular injury. (Circulation. 2009;120:785-791.)

Key Words: platelets ■ arteriosclerosis ■ atherosclerosis ■ thrombosis ■ platelet-derived factors ■ carotid arteries

Vascular injury is a critical step in the pathogenesis of coronary artery disease. Platelets become activated at sites of vascular injury and secrete their α- and dense-granule contents. Platelet α-granules are the primary storage organelle for adhesive and proinflammatory molecules, such as P-selectin, CD40L, and RANTES,1–2 but platelet dense granules also contain cell-activating molecules (eg, serotonin, histamine, and adenine nucleotides) that may be considered to be proinflammatory.3 Under pathophysiologic conditions, local delivery of adhesive and proinflammatory molecules released from platelet granules may contribute to arteriosclerosis and neointima formation after injury.4,5

Clinical Perspective on p 791

Previous studies have used antplatelet agents to examine the role of platelets in arterial wall remodeling after injury. Aspirin, at doses that reduce platelet aggregation, had no effect on neointimal remodeling after acute injury in animal models.6,7 Clinical studies using P2Y12 receptor antagonists show that these agents do not reduce rates of restenosis after stent placement or angioplasty.8–10 Data on the role of IIb/IIIa inhibitors remain controversial. Several clinical studies found treatment with IIb/IIIa inhibitors reduced the need for target-vessel revascularization over conventional treatment,11,12 whereas other studies found no benefit for restenosis.13,14 Although these antplatelet agents may be used to determine the role of specific platelet activators or platelet aggregation (IIb/IIIa inhibitors), they cannot be used to directly address the role of platelet secretion in vessel remodeling after acute injury.

To determine the contributions of platelet dense-granule secretion to thrombosis and atherosclerotic vascular remodeling, we used atherosclerosis-prone apolipoprotein E–deficient (ApoE−/−) mice with defective platelet dense-granule secretion due to a mutation in the Hermansky-Pudlak syndrome 3 (HPS3) gene that results in a functional gene
knockout or deletion of HPS3 mRNA (ie, HPS3<sup>−/−</sup>).<sup>15–17</sup> HPS3-deficient mice have impaired platelet dense-granule secretion due to defects in the biogenesis of these organelles.<sup>15–17</sup> We hypothesized that compared with atherosclerotic mice with normal platelet secretion (ApoE<sup>−/−</sup>, HPS3<sup>+/−</sup>), mice with impaired dense-granule secretion (ApoE<sup>−/−</sup>, HPS3<sup>−/−</sup>) would have attenuated thrombotic occlusion, vascular inflammation, and atherosclerotic remodeling after injury.

**Methods**

**Animals**

Mice (ApoE<sup>−/−</sup>, HPS3<sup>−/−</sup>) were obtained from the Jackson Laboratory (Bar Harbor, Me) and were congenic with the C57/B6 line. Animals were housed and cared for in accordance with the “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare [Department of Health and Human Services] National Institutes of Health publication No. 85-23, revised 1996). Procedures that involved laboratory animals were approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia and the Standing Committee on the Use of Animals in Research and Teaching at Harvard University. For cholesteryl assays, carotid injury model, and analysis of atherosclerosis, mice were placed on a high-fat diet (TD.88137 adjusted calorie proportion of CD41- and CD45-positive events in 5000 leukocyte-gated events) were identified with anti-CD41 antibodies (see above) and anti-CD45 antibodies (BD Pharmingen). Isotype-matched control antibodies were used to measure nonspecific binding. The percent of platelet-leukocyte aggregates was determined by assessing the proportion of CD41- and CD41-positive events in 5000 leukocyte-gated events with the following formula: % Leukocytes in platelet-leukocyte events=100×(CD41<sup>+</sup>,CD45<sup>−</sup>/CD41<sup>−</sup>,CD45<sup>+</sup>+CD41<sup>+</sup>,CD45<sup>−</sup>/CD41<sup>−</sup>,CD45<sup>+</sup> cells+CD41,CD45 double-positive cells). Differences between groups were assessed by a Mann–Whitney U test.

**Platelet Aggregation Assays**

Aggregation studies were performed with isolated platelets or in whole blood with a lumiaggregometer (Chrono-Log, Havertown, Pa) according to the manufacturer’s instructions. To isolate the platelets from other blood cells, anticoagulated blood was diluted 2-fold with modified Tyrode’s buffer and centrifuged at 150g for 5 minutes. The platelet-rich upper phase was transferred to a fresh tube, and manual platelet counts were performed with a hemocytometer. Equivalent platelet counts were obtained by the addition of platelet-poor plasma. The final platelet count was adjusted to 1.5×10<sup>10</sup> cells/mL with modified Tyrode’s buffer. The samples were kept in a 37°C incubator and used within 2 hours. Chrono-Lume reagent (Chrono-Log) was added to the samples to measure ATP secretion by luminescence, whereas aggregation was measured by light transmission. Differences between groups were assessed with the Mann–Whitney U test.

**Electron Microscopy**

Platelets (in platelet-rich plasma) were fixed with 4% paraformaldehyde/0.2% glutaraldehyde for 1 hour on ice. After they were washed with PBS, pellets were dehydrated with graded ethanol steps and embedded in LR White resin (Electron Microscopy Science, Hatfield, Pa). Ultrathin sections were stained with uranyl acetate and lead and viewed on a JEOL 1010 transmission electron microscope (JEOL USA, Peabody, Mass). α-Granules were counted by an investigator blinded to the genotype of the specimen. Differences between groups were assessed with the Mann–Whitney U test.

**Cardiot Injury Model**

Ferric chloride–induced arterial injury was performed according to published procedures.<sup>21</sup> After the animal was anesthetized adequately with isoflurane (1.5% to 2%), a midline incision was made in the neck, and the left common cardiot artery was exposed by blunt dissection. Baseline blood flow was measured with a miniature Doppler flow probe (model 0.5SPB; Transonic Systems, Ithaca, NY) connected to a TS420 flow module (Transonic Systems). The data were recorded and analyzed with the PowerLab 16/30 data acquisition unit and Chart software (AD Instruments, Colorado Springs, Colo). After baseline blood flow was established, the carotid artery...
was rinsed carefully with sterile saline prewarmed to 37°C to remove any trace of acoustic couplant (SurgiLube, Fougera, Melville, NY). Thrombosis was induced by application of 2 pieces of filter paper (1×2 mm) saturated with 5% ferric chloride (Sigma, St Louis, Mo). The pieces of filter paper were placed on opposite sides of the carotid artery (above and beneath) in contact with the adventitial surface of vessel. Care was taken to avoid regions with obvious atherosclerotic plaques. The filter paper was applied for 3 minutes and then removed. Carotid blood flow was monitored at time 0 (before the ferric chloride paper application) and continuously for 30 minutes after application of the filter paper. Differences in carotid blood flow between groups were assessed by a Mann–Whitney U test. After the 30-minute monitoring period, vessels in the deeply anesthetized animals were perfusion-fixed with 4% paraformaldehyde.

For remodeling studies, the surgical opening was rinsed with sterile saline, and the muscle and skin layers were closed with 6-0 sutures. The animal was kept on a 37°C heating pad until it was conscious and active (usually 2 hours), then it was placed in our animal housing facility and monitored daily. After 3 weeks, the animal was deeply anesthetized with isoflurane (1.5% to 2%), and vessels were perfusion-fixed as above.

**Histology**

For histological analysis, paraformaldehyde-fixed carotid vessels were processed and embedded in paraffin. Cross sections (4 μm) were cut from paraffin blocks and mounted on treated slides (Superfrost Plus, VWR Scientific Products, Suwanee, Ga). Equally spaced cross sections were stained with Verhoeff-van Gieson stain. Digital images were captured on a Zeiss AXIOPHOT upright microscope with Axiocam camera (Carl Zeiss Microimaging, Thornwood, NY). Analysis was performed by an investigator blinded to the genotypes of the specimens. Intima was defined as the area bounded by the endothelium and internal elastic lamina. Media was defined as the area bounded by the internal and external elastic lamina. Intimal and medial areas of each cross section (typically 10 sections) were quantified with National Institutes of Health ImageJ software and summarized into a mean area for each artery. The means of the mean intimal and medial areas for arteries in each group were compared by an unpaired Student t test.

**Immunohistochemistry**

Cross sections (4 μm) were cut from paraffin blocks and mounted on treated slides (Superfrost Plus, VWR Scientific Products). Slides were air-dried overnight, then placed in a 60°C oven for 30 minutes. Slides were deparaffinized and run through graded alcohols to 100% alcohol. Sections were quenched with 0.3% H2O2 for 5 minutes, followed by distilled water rinse. Endogenous peroxidases were blocked with 3% H2O2 in 0.05 M Tris buffer, pH 6.0 (Dako Corp, Carpinteria, Calif) with a steamer, followed by a distilled water rinse. Slides were then counterstained with hematoxylin (Richard-Allan Science, Kalamazoo, Mich) at 1:100 dilution for 1 hour, followed by 2 rinses with distilled water. Immunostaining was assessed by flow cytometry in blood from HPS3−/− mice and congenic wild-type (HPS3+/+) mice (gray bars). The percent of leukocyte-platelet aggregates (LPA %) was assessed by flow cytometry (n=4 pairs of mice). G. Secretion of the α-granule protein P-selectin by platelets after stimulation in whole blood with the calcium ionophore A23187 (0 or 2 μmol/L). Platelet P-selectin expression (percent activation) was measured by flow cytometry in blood from HPS3−/− mice (black bars) and congenic HPS3−/+ mice (gray bars). Platelet responsiveness to ADP was retained.

**Data Analysis**

Statistical analyses were performed as indicated above, and differences between groups were considered significant if P<0.05. The number of animals analyzed, n, is given in the figure legends. Data are reported as mean±SEM.

**Results**

Previous studies have established that the secretion of platelet dense-granule contents such as ATP and serotonin is markedly reduced in HPS3−/− mice.17 In confirmation of these findings, we found that secretion of ATP was reduced (30-fold) in HPS3−/− mice reconstituted with ADP doses that do not induce secretion in wild-type congenic HPS3−/+ mice (Figure 1A through 1C), which indicates that responsiveness to ADP was retained.
Table. Parameters After 16 to 18 Weeks on a High-Fat Diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ApoE−/−, HPS3+/+</th>
<th>ApoE−/−, HPS3−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, females, g</td>
<td>27±1</td>
<td>26±2</td>
</tr>
<tr>
<td>Body weight, males, g</td>
<td>37±8</td>
<td>37±6</td>
</tr>
<tr>
<td>Fasting cholesterol, mg/DL</td>
<td>950±140</td>
<td>940±150</td>
</tr>
</tbody>
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Compared with wild-type mice, HPS3−/− mice had equivalent platelet counts (1.25±0.18x10^11 and 1.35±0.11x10^10 platelets per mL, respectively). Electron microscopic analysis of ultrathin sections revealed that HPS3−/− mice also had comparable numbers of α-granules (4.65±1.72 granules per platelet; Figure 1D) with a morphology similar to congenic control mice (4.32±2.09 granules per platelet; Figure 1E). Consistent with this expression of the α-granule, protein P-selectin expression was the same in wild-type and HPS3−/− platelets after maximal cell activation by calcium ionophore (Figure 1G) or by thrombin receptor peptide and calcium ionophore, which are known to induce maximal platelet secretion (not shown). However, leukocyte-platelet interactions were reduced in HPS3−/− mice compared with wild-type mice (Figure 1F). Although previous reports have indicated that bleeding times are prolonged in HPS3−/− mice, coagulation parameters were the same in HPS3−/− and normal mice as assessed by measurements of prothrombin times (8.0±0.3 versus 7.9±0.3 seconds, respectively; n=7) and partial thromboplastin times (23.3±2.0 versus 23.8±2.1 seconds, respectively; n=10).

To examine the effect of platelet dense-granule secretion on thrombosis and vascular remodeling in atherosclerosis, ApoE−/−, ApoE−/− mice were compared with ApoE−/−, HPS3+/− mice. Mice were placed on a high-fat diet beginning at 6 weeks of age to accelerate the development of atherosclerotic lesions. ApoE−/−, HPS3+/− mice exhibited similar cholesterol levels and weight as control mice (ApoE−/−, HPS3+/−; Table) after 10 to 13 weeks on a high-fat diet. Microscopic analysis confirmed that the mice developed atherosclerosis after 16 to 18 weeks on this high-fat diet.

To examine the effects of platelet secretion on arterial thrombosis, carotid arteries were treated with FeCl3 after mice had been on the high-fat diet for 16 to 18 weeks. Occlusion of the carotid artery occurred in less than 10 minutes in all ApoE−/−, HPS3+/− mice (Figure 2C). Microscopic examination confirmed that this was due to thrombotic occlusion (Figure 2A). In contrast, none of the ApoE−/−, HPS3−/− mice experienced a decrease in blood flow due to thrombotic occlusion during the 30-minute study period (Figure 2C). The area under the curve of the relative Doppler blood flow versus time showed that ApoE−/−, HPS3−/− mice were protected against arterial thrombotic occlusion (Figure 2C; P<0.01). No thrombotic occlusion was obtained in ApoE−/−, HPS3−/− mice even with high-dose (15%) FeCl3 (Figure 2C). Histological examination confirmed the absence of an occlusive thrombus in the arteries of ApoE−/−, HPS3−/− mice (Figure 2B).

The effect of arterial injury on atherosclerotic remodeling was assessed 3 weeks after the injury. In the injured arteries, ApoE−/−, HPS3+/+ mice displayed significantly greater intimal hyperplasia (Figure 3A) than ApoE−/−, HPS3−/− mice (Figure 3B; P<0.001). In contrast, minimal (if any) intimal hyperplasia was observed in the contralateral, uninjured (control) arteries of either the ApoE−/−, HPS3+/+ mice or the ApoE−/−, HPS3−/− mice (Figure 3A and 3B). There was no significant difference in the medial areas between the 2 groups. When assessed by intima-to-media ratios, FeCl3-induced injury produced a 14-fold increase in the intima-to-media ratio in the ApoE−/−, HPS3+/+ mice (Figure 3C); however, injury-induced arterial remodeling, assessed by intima-to-media ratios, was reduced >65% in ApoE−/−, HPS3−/− mice compared with ApoE−/−, HPS3+/+ mice (P<0.05; Figure 3C).

Microscopic examination revealed that the cell population in the neointima was heterogeneous in both mouse genotypes, as shown by immunostaining for smooth muscle cells (α-smooth muscle actin), macrophages (Mac-3), and leukocytes (CD45). Although the lesions differed in size, smooth muscle cells were abundant in the neointima for both ApoE−/−, HPS3−/− and ApoE−/−, HPS3−/− mice (Figure 4). Immunostaining for the mouse common leukocyte antigen, CD45, showed that there was significantly less inflammatory cell accumulation within the neointima in ApoE−/−, HPS3−/− mice. In ApoE−/−, HPS3+/+ mice, 30.5±4.0% of neointimal cells were CD45-positive, whereas only 8.0±3.6% were CD45-positive in ApoE−/−, HPS3−/− lesions (n=5, P<0.01).
Immunostaining with the activated macrophage marker Mac-3 revealed that the inflammatory cells consisted primarily of macrophages. The neointima consisted of 25.2±7.7% Mac-3–positive cells in the ApoE−/−, HPS3+/+ mice and only 6.9±1.9% Mac-3–positive cells in ApoE−/−, HPS3−/− mice (n=5, P<0.05).

Discussion

Mice with ApoE gene deletion develop atherosclerosis that is marked by a mild prothrombotic phenotype, with lesions similar to those seen in humans.24,25 After ferric chloride injury, ApoE−/− mice, especially those on a high-fat diet, have enhanced neointimal hyperplasia that contributes to luminal stenosis.25–27 This process involves migration of smooth muscle cells from the media across the internal elastic lamina, proliferation in the neointima, and deposition of extracellular matrix.28 Inflammatory cells (eg, leukocytes and macrophages) also contribute to the atherosclerotic, vascular remodeling process. Because platelets secrete potent cell-activating agents at sites of vascular injury, we hypothesized that impaired dense-granule secretion might protect against arteriosclerosis after arterial wall injury. Consistent with this notion, we found that thrombotic occlusion and atherosclerotic remodeling were markedly attenuated in the injured vessels from mice with defective platelet dense-granule secretion due to deficiencies of HPS3. The reduced atherosclerotic remodeling was associated with marked reductions in neointimal size and content of α-actin–positive smooth muscle cells, CD45–positive leukocytes, and Mac-3–positive macrophages.

The defective secretion of HPS3-deficient platelets is attributed to a dense-granule storage pool disorder because these platelets lack adenine nucleotides and other molecules typically found in dense granules.15,17 However, ultrastructural studies suggest that platelet α-granule numbers and morphology are normal in HPS3-deficient platelets, as is their capacity to secrete the α-granule marker P-selectin (Figure 1). In addition, coagulation parameters (prothrombin time and partial thromboplastin time) are normal in HPS3-deficient mice. Still, HPS3 deficiency markedly inhibits thrombosis in response to FeCl3-induced injury. Indeed, unlike previous studies with ADP-receptor antagonists, aspirin, or other agents, we could not overcome the antithrombotic effects of HPS3 deficiency even with high doses of FeCl3 (up to 15%).21

Diminished secretion of platelet dense-granule contents may specifically affect vascular remodeling after injury. Molecules secreted from platelet dense granules may act directly on cell types that are involved in the growth of neointima after acute injury. In vitro, molecules found in dense granules have been shown to have direct effects on cells involved in atherosclerotic remodeling. Serotonin has a mitogenic effect on endothelial cells29 and smooth muscle cells; histamine and serotonin increase vascular permeability; and ADP increases the agonist-induced oxidative burst in

![Figure 3](image-url)

**Figure 3.** Reduced arteriosclerotic vessel remodeling 3 weeks after FeCl3-induced injury in HPS3−/− mice. A, Representative Verhoeff-Van Gieson–stained cross sections from injured carotid arteries or uninjured contralateral control arteries taken 21 days after injury. Images originally taken at 200× magnification. B, Ratios of intima to media from cross sections of FeCl3-injured arteries and contralateral control arteries 21 days after experimental injury. n=5 mice per group.

![Figure 4](image-url)

**Figure 4.** Reduced inflammation after FeCl3-induced injury in ApoE−/−, HPS3−/− mice. Cross sections of carotid arteries 21 days after FeCl3 injury were immunostained for smooth muscle α-actin, macrophages (Mac3), and leukocytes (CD45). All images were initially taken with 400× magnification. For ApoE−/−, HPS3+/− mice, the neointimal lesions were small, and arrows indicate the internal elastic lamina to demarcate the boundaries of the lesions. For ApoE−/−, HPS3−/− mice, the large neointimal lesions completely fill the figure.
polymorphonuclear leukocytes and amplifies proliferation of smooth muscle cells. Platelet granule secretion may accelerate atherosclerotic remodeling by delivering high concentrations of these molecules to the cells that form the neointima.

The thrombus itself also may play a role as an organizing matrix in neointima formation. Fibrin is an important determinant of neointima formation, providing a network to support smooth muscle cell migration. Endothelial denudation activates platelets to form a thrombus. However, only minimal, nonocclusive mural thrombi formed in ApoE−/−, HPS3−/− mice, probably because HPS3 deficiency interferes with the amplification of the platelet activation that normally occurs with dense-granule secretion. The adhesive receptors on activated platelets present in the thrombus play a role in recruiting inflammatory cells (neutrophils, monocytes, and lymphocytes) to the injured area through P-selectin secretion and other mechanisms; this has been shown to accelerate atherosclerotic lesion development. Leukocyte-platelet interactions are reduced in ApoE−/−, HPS3−/− mice. In addition, as noted above, the diminished dense-granule secretion in ApoE−/−, HPS3−/− mice may also decrease leukocyte activation, and this may also affect vascular remodeling. Once recruited into the vessel wall, macrophages contribute to local inflammation through the secretion of inflammatory mediators, including adhesion molecules, cytokines, chemokines, matrix metalloproteases, and oxygen radicals. Therefore, a lack of thrombus and/or alterations in cellular signaling or other mechanisms could prevent the recruitment of inflammatory cells and migration of smooth muscle cells in the injured vessel wall and contribute to the diminished vascular remodeling that occurs in ApoE−/−, HPS3−/− mice.

Only small numbers of humans have been described with decreased platelet dense-granule secretion. Some individuals with altered platelet dense-granule secretion alone have been found to have increased circulating levels of serotonin with pulmonary hypertension. However, among humans and mice with decreased dense-granule secretion in the setting of Hermansky-Pudlak syndrome, there is significant variance according to the genetic cause. Individuals with HPS1 appear to be at risk for restrictive pulmonary disease, however, humans and mice with HPS3 deficiency appear to have minimal other physiological abnormalities, if any.

The present findings indicate that platelet secretion reduces thrombosis and subsequent vascular remodeling after injury. Whether platelet secretion affects vascular remodeling through mechanisms independent of its effects on thrombosis cannot be determined solely through vascular injury models. However, a previous study suggested that some mice with platelet-secretion disorders had reduced susceptibility to diet-induced atherosclerosis. It will be important to further elucidate these mechanisms and to explore the potential utility of platelet dense-granule secretion as a therapeutic target in atherothrombotic disease.

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Disclosures
None.

References
In patients with atherosclerosis, platelets contribute to myocardial infarction, ischemic stroke, and peripheral vascular ischemia. Platelets secrete molecules from dense granules that activate other platelets, vascular cells, and leukocytes, but the contribution of platelet dense-granule secretion to thrombotic arterial occlusion and vascular remodeling after injury is not well understood. We examined thrombotic occlusion and vascular remodeling after injury in atherosclerotic mice with normal and decreased platelet dense-granule secretion. Mice with decreased platelet dense-granule secretion showed normal aggregation responsiveness to ADP, but platelet-leukocyte interactions were reduced. Mice with decreased platelet secretion were completely protected against thrombotic arterial occlusion. Three weeks after thrombosis, mice with impaired dense-granule secretion were also protected against vascular inflammation and neointimal hyperplasia, a type of vascular remodeling typically seen after vascular injury. In summary, in atherosclerotic mice, reduced platelet dense-granule secretion could be a target for future therapeutic agents.
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