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Regulation of tissue factor-induced coagulation and platelet aggregation in flowing whole blood

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Summary

Photochemically induced thrombosis (a thrombin-dependent process) was measured in rats treated with moderate doses of anticoagulants, but which appeared to be unchanged. We considered the possibility that platelet-inhibiting agents, which also indirectly inhibit coagulation, would act as more potent antithrombotic agents. Inhibitors used as such were prostaglandin E_1 (PGE_1), which elevates cyclic AMP levels, and the $P2Y_{12}$ ADP-receptor antagonist, AR-C69931MX. Effects of these agents were investigated in an *ex vivo* model system, in which whole blood under coagulant conditions was perfused over fibrinogen at defined wall shear rate. Perfusion of blood (rat or human) in the presence of tissue factor resulted in deposition of activated platelets and subsequent aggregate formation, along with exposure of procoagulant phosphatidylserine (PS) on the platelet surface and formation of fibrin fibers. In the presence of PGE_1 aggregation was completely inhibited, but platelet adhesion and PS exposure were only party reduced, while fibrin formation was hardly affected. Treatment with AR-C69931MX caused similar, but less complete effects. These results indicate that in tissue factor-triggered blood under conditions of flow: (i) the platelet procoagulant response is independent of aggregate formation; (ii) the platelet-inhibiting effect of PGE_1 and AR-C69931MX is sufficient to suppress aggregation, but not platelet adhesion and coagulation. These platelet inhibitors thus maintain their aggregation-inhibiting effect at sites of thrombin formation.

Keywords

Coagulation, platelets, prostaglandin, thrombin, thrombus

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Introduction

Haemostasis involves formation of a vaso-occlusive platelet plug and coagulation of blood plasma. Although usually studied separately, it is known that platelet activation and the formation of thrombin in the coagulation cascade are mutually dependent processes. The extrinsic coagulation pathway is initiated by tissue factor (TF), either blood-borne or exposed at the surface of a damaged vessel wall (1). Thrombin, once formed from prothrombin, has multiple amplifying effects. It facilitates its own generation by activating the coagulation factors V, VIII, and XI. Thrombin converts fibrinogen into fibrin and, furthermore, stimulates platelets to change shape and assemble into aggregates and fibrin-containing thrombi.

Platelets, in turn, support and maintain the coagulation process in different ways. They bind coagulation factors like prothrombin and factor XI (2, 3), and they secrete coagulation factors when stimulated by agonists that induce rises in cytosolic $[Ca^{2+}]_i$. Activated platelets also expose procoagulant phosphatidylserine (PS) at their outer membrane surface (4, 5), which serves as a site for the assembly and activation of tenase and prothrombinase complexes, forming factor Xa and thrombin, respectively (6–8). Exposed PS thereby is the main binding site on platelets for the γ-carboxyglutamate-containing enzymes, factor IXa and factor Xa (8, 9). The significance of PS exposure becomes apparent in patients suffering from Scott syndrome, a rare bleeding disorder in which platelets are partly deficient in PS exposure upon activation (10, 11).

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Platelet-rich plasma (PRP) triggered with TF enters a potent reinforcement loop of thrombin generation, fibrin formation and platelet activation (12, 13). In such a plasma system, platelet-dependent thrombin generation can be suppressed by antibodies against the platelet-adhesive receptors, integrin αIIbβ3 (fibrinogen receptor) and glycoprotein Ib/IX/V (von Willebrand factor receptor) (14, 15). Similarly, thrombin generation is reduced in the PRP from patients with Glanzmann's thrombasthenia (deficient in αIIbβ3) or von Willebrand disease (deficient in von Willebrand factor) (12, 15). Together, this suggests that the mutual interaction of platelets and coagulation is of physiological importance in hemostasis, and that these glycoproteins herein play an important role.

A number of platelet inhibitors can also suppress thrombin generation and coagulation in platelet-containing plasma. Particularly active in this respect are cyclic AMP elevating agonists, such as prostaglandin E_1 (PGE_1), and $P2Y_{12}$ receptors antagonists like AR-C69931MX (12, 16, 17). The latter receptor comprises the pharmacological target of clopidogrel and ticlopidine, and contributes to ADP-mediated platelet aggregation (18–22). As these compounds also downregulate integrin αIIbβ3 activation and platelet aggregation, it is possible that their effects on thrombin generation are via regulation of integrin activation.

The rat is a laboratory animal widely used to study thrombosis *in vivo*. Photochemical injury of the rat femoral artery is an established model for thrombosis with limited invasive effect, which relies on endothelial damage and subsequent initiation of coagulation and platelet activation (23, 24). In earlier *in vitro* studies with platelet-containing plasma, we have established that, also in rat, thrombin generation triggered by the TF pathway relies on the activation of platelets and the coagulation system (25, 26). If this interaction of platelets and coagulation is of (patho)physiological relevance, it is expected that thrombin inhibitors would be effective in such a thrombosis model.

In the present paper, we studied the effect of thrombin inhibition on photochemically induced thrombosis tendency in this animal model. To this end, rats were subjected to moderate treatment with heparin or warfarin. The inability of these anticoagulants to influence the thrombotic process prompted us to investigate the effects of the anti-platelet agents, PGE_1 and AR-C69931MX, which indirectly affect coagulation via their platelet-inhibiting activity. To mimic physiological conditions, we studied the effects of these agents in whole blood triggered with tissue factor on the formation of platelet-fibrin thrombi under conditions of flow and coagulation.

Methods

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA, USA). Bovine fibrinogen, bovine serum albumin (BSA), MRS 2179, PGE_1, rat thrombin, rose bengal and unfractionated heparin were from Sigma (St. Louis, MO, USA). Recombinant human TF was from Dade (Düdingen, Switzerland); Oregon green 488 (OG488)-labeled and unlabeled annexin A5 (annexin V) from Nexins Research (Hoeven, The Netherlands); benzyloxycarbonyl Gly-Gly-Arg 7-amido-4-methylcoumarin (Z-GGR-AMC) and Gly-Pro-Arg-Pro (GPRP) from Bachem (Bubendorf, Switzerland); Fluo-3 acetoxymethyl ester and human OG488-fibrinogen from Molecular Probes (Leiden, The Netherlands). Collagen ('Horm' type I) was from Nycomed (Munich, Germany). Warfarin (coumarin) was bought from Dupont Merck and heparin (enoxaparin) from Rhone-Poulenc Rorer. FITC-labeled monoclonal antibody PAC1, against the activated human αIIbβ3 was obtained from Becton and Dickinson. AR-C69931MX was kindly provided by AstraZeneca R&D (Charnwood, UK), Other materials were from sources previously described (25).

Animals

Forty eight male Wistar rats (Charles River, Sulzfeld, Germany) were used for the intervention study. Of these, 24 rats were subjected to photochemically induced thrombosis; 24 other rats were used for *ex vivo* measurements. In each cohort, 3 groups of 8 rats (selected at random) were formed. The animals were subjected to one of the following interventions: none (control group), heparin (2 mg enoxaparin kg^{-1}, subcutaneous injection at 49, 25 and 1 h before experiment), or warfarin (0.3 mg kg^{-1}, intravenous injection at 24 h before experiment). Subsequent experiments were carried out with blood from adult Wistar rats. The animals were anesthetized by intra-peritoneal injection with Nembutal (50 mg kg^{-1} body weight) and subjected to abdominal aortic puncture. Animals were sacrificed after blood collection (10–12 mL). The studies were approved by institutional animal care committees (Unilever Research Vlaardingen and University of Maastricht).

In vivo thrombus formation

Rats were subjected to a standard protocol for measurement of photochemically induced thrombosis (27). Briefly, the rats were anaesthetized with ketamine and domitor (intramuscular, 40 and 500 mg kg^{-1} body weight, respectively), and fixed on a heating pad at 36°C. The femoral artery and vein were separated from surrounding tissue, and a catheter was inserted through the jugular vein. An ultrasound flow probe was placed against the femoral artery to measure blood flow. Green light (540 nm) from a xenon lamp was led through an optical fiber positioned at 5 mm from a part of the femoral artery proximal to the flow probe. Light was switched on after stable flow for 3 min; 1 min later, rose bengal (10 mg kg^{-1}) was infused over a period of 1.5 min; at 4 min after rose bengal infusion the light was switched off. Blood flow was recorded during 30 min. In this model, endothelial injury and thrombosis are caused by free radical formation via a photochemical reaction between rose bengal and green light (23). Thrombus formation was evaluated by measuring the time to vascular occlusion and the area under the curve of blood flow, calculated over 30 min. The latter gives an indication of the overall blood flow response during the whole measurement period (27).

Collection and preparation of rat and human blood

For most experiments, rat blood was collected into 0.1 vol of 129 mM trisodium citrate. The blood was used as such, or centrifuged to obtain platelet-rich plasma (PRP) and platelet-free plasma (PFP) (26). To prepare Fluo-3-labeled platelets, blood was collected into 0.2 vol of 183 mM glucose, 80 mM trisodium acetate

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and 52 mM citric acid. Washed platelets (5×10^8 mL^{-1}) were loaded with 5 μM Fluo-3 acetoxymethyl ester in the presence of 0.5 g L^{-1} Pluronic F-127 for 45 min (room temperature). The platelets were suspended at 1×10^9 mL^{-1} in Hepes buffer pH 7.45 (in mM: NaCl 136, glucose 5.6, Hepes 5, KCl 2.7, $MgCl_2$ 2, NaH_2PO_4 0.42, and 0.1% BSA), and re-added to citrate-anticoagulated blood from the same animal to give a fraction of 2% labeled platelets.

Human blood was collected from healthy volunteers into 0.1 vol of 129 mM trisodium citrate. For some experiments PRP and (defibrinated) PFP were prepared, as described elsewhere (15).

Coagulation factors and platelet aggregation

In rat PFP, prothrombin time, and activities of fibrinogen, prothrombin, factors VII and antithrombin were measured as previously described, and the anticoagulant effect of heparin in rat plasma was quantified from the anti-thrombin activity (26). Platelet aggregation in citrated whole blood was measured as change in impedance, while stirring at 37°C in a Chronolog whole blood aggregometer (Chronolog Corporation, Havertown, PA, USA) (28). Platelet aggregation in recalcified PRP under coagulant conditions was measured by turbidometry with/without 2 mM GPRP in siliconized cuvettes, while stirring at 37°C.

Thrombin generation

Thrombin generation was measured in freshly obtained rat or human PRP using low-affinity fluorescent substrate, Z-GGR-AMC (0.42 μM), following the procedure described for human plasma (29). PRP (1.0×10^8 platelets mL^{-1}, f.c.) was triggered with 0.5–2 pM TF and 16.7 mM $CaCl_2$ in 96-well microtitre plates (37°C). Fluorescence from cleaved amidomethyl coumarin was followed in time, and first-derivative curves were constructed. The curves were converted to nanomolar thrombin by using internal calibrator (26).

Perfusion of whole blood under coagulant conditions

Rat or human whole blood was perfused over a glass surface covered with fibrinogen, which is an adhesive protein abundantly present in plasma and involved in platelet-platelet contact. Immobilized fibrinogen, as an excellent starting point for fibrin fiber formation, was used for the detection of initial stages of coagulation. Fibrinogen-coated coverslips were blocked with Hepes buffer pH 7.45 containing 2% (w/v) BSA (5). The coverslips were mounted in a transparent, parallel-plate flow chamber (9). The chamber (flow-cell volume of 60 μL) was connected, via siliconized plastic tubes and a three-way adapter, to plastic syringes placed in two pulse-free perfusion pumps (Harvard Apparatus, South Natick, MA, USA). Pump 1 contained a syringe filled with blood (normal perfusion rate of 27 mL h^{-1}). Pump 2 contained a syringe with 200 mM $CaCl_2$, 153 mM NaCl and 20 pM TF (perfusion rate 3 mL h^{-1}). Together, these pumps produced a flow rate of 30 mL h^{-1}, resulting in a wall shear rate at the flow chamber surface of 250 s^{-1}. Other experiments were carried out at 1500 s^{-1} by increasing the rate of pumping. Experiments were stopped before occlusion of the flow chamber by clotting. Rinsing was at the same flow rate using heparinized Hepes buffer pH 7.45 containing, as indicated, 2 mM $CaCl_2$ and 0.5 μg L^{-1} OG488-annexin A5.

Phase-contrast and fluorescent images were taken from the perfusion chamber, placed on the stage of an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). Intensified, charge-coupled device cameras were connected to the microscope via a beam splitter (5). Digital images were obtained with a recording system controlled by Quanticell software (Visitech, Sunderland, UK). Excitation and emission wavelengths were 485±11 and 530±15 nm, respectively.

Changes in Fluo-3 fluorescence from single platelets were converted into pseudo-ratio values and converted into nanomolar concentration of cytosolic $[Ca^{2+}]_i$ as described (30). Fluorescence and phase-contrast transmission images (10 microscopic fields per experiment) were analyzed on coverage by platelets or label using Quanticell and ImagePro software, respectively (9).

Statistics

Differences between intervention groups and the control group were analyzed with the Dunnet's test (mean square error) taking into account all values obtained. Effects of antagonists were compared to control condition with a Mann-Whitney U-test (SPSS, Chicago, IL, USA). Data are mean values ± SEM.

Results

Effect of moderate anticoagulation treatment on *in vivo* thrombosis tendency in rats

Thrombus formation involves platelet activation and coagulation, which are mutually stimulatory processes, both in human and rat (13, 26). To study the importance of this interaction, we used a limited invasive thrombosis model in rats, which relies on photochemically induced exposure of tissue factor (TF) and subsequent activation of coagulation and platelets (23, 31). In a standardized way, thrombus formation was induced in the femoral artery by infusion of rose bengal and controlled illumination with green light (27). To achieve moderate anticoagulation, the rats were injected at 1–2 days before the experiment with intermediate doses of heparin (enoxaparin, a low molecular-weight heparin increasing the activity of antithrombin) or warfarin (inhibiting the synthesis of vitamin K-dependent prothrombin, factors VII, IX and X and proteins C and S). As shown in Table 1, intervention with heparin or warfarin led to a slight, insignificantly prolonged occlusion time of the artery.

The efficacy of the treatments was verified using blood samples from rats that were similarly treated, but not used for thrombosis induction. Thrombin generation was measured in citrated PRP, triggered with TF/$CaCl_2$, using the thrombogram method. The endogenous thrombin potential (ETP, area-under-the-curve) was substantially decreased by heparin and warfarin treatment with 87% and 52.5%, respectively (Table 1). Platelet aggregation, measured in anticoagulated whole blood was not significantly changed. After heparin treatment, levels of coagulation factors in plasma were unaltered, whereas increased antithrombin activity was detected. After warfarin treatment, levels of the vitamin K-dependent coagulation factors prothrombin and factor VII were reduced by 40–60%. This was accompanied by a prolonged prothrombin time, indicative of delayed coagulation. Taken together, these moderate anticoagulant treatments led to a

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	Control	Heparin	Warfarin
Thrombosis tendency			
– time to occlusion (min)	17.4±3.6	19.7±3.5	21.5±3.1
– area under flow curve (AU$^+$)	1160±241	1293±128	1361±213
Thrombin generation in PRP			
– time to peak (min)	8.63±0.25	22.4±0.78**	10.2±0.65
– peak height (AU)	36.6±1.3	3.62±1.4**	18.3±4.2**
– ETP (AU × min)	186±8.3	23.6±8.9**	88.4±23**
Whole blood aggregation			
– (% aggregation min^{-1})	39.7±4.2	31.1±4.8	31.1±7.5
Prothrombin time (% of pool)	99.1±1.7	98.6±2.1	75.4±8.6*
Fibrinogen (g L^{-1})	2.19±0.04	2.27±0.06	2.27±0.07
Prothrombin (% of pool)	96.9±2.1	91.4±2.0	54.5±7.6**
Factor VII (% of pool)	95.6±5.5	85.5±3.2	36.6±9.6**
Antithrombin (% of pool)	107±2.5	106±2.8	105±3.1
Thrombin inhibiting activity (mol^{-1} s^{-1})	n.d.	53009±3000	n.d.

Table 1: Effect of heparin or warfarin intervention on thrombosis tendency and hemostatic parameters in rat. Animals were untreated (control) or subjected to intervention with heparin (2 mg kg^{-1} enoxaparin, injection at 49, 25 and 1 h before measurement) or warfarin (0.3 mg kg^{-1}, injection at 24 h before measurement). Thrombosis tendency was measured in the femoral artery following rose bengal injection and photochemical induction. Other rats were subjected to the same interventions, and blood was taken on citrate to determine: thrombin generation in PRP (triggered with 16.7 mM $CaCl_2$ and 1 pM TF), giving time to thrombin peak, thrombin peak height and endogenous thrombin potential (ETP); whole blood aggregation (1 µg mL^{-1} collagen); prothrombin time; individual coagulation factors (data as percentages of rat pool plasma). Mean ± SEM (n=8); *P<0.01 and **P<0.001 compared to control. $^+$AU, arbitrary units; n.d., not determined.

marked suppression in thrombin generation which, apparently, was not sufficient to significantly reduce the *in vivo* thrombosis tendency.

Earlier studies with human PRP showed that the platelet-inhibiting agents, PGE_1 (which elevates cyclic AMP) and AR-C69931MX (which specifically blocks $P2Y_{12}$ receptors for ADP), caused a substantial decrease in thrombin generation (12, 16), which was similar in size as detected in the rats subjected to the anticoagulant interventions. This raised the question of possible advantages of such agents, which combine antiplatelet with indirect anticoagulant activity, in comparison to moderate anticoagulant treatment. We investigated this under a variety of physiologically relevant conditions.

In vitro effects of platelet inhibitors on thrombin generation and aggregation in rat plasma

First we determined the effects of PGE_1 and AR-C69931MX in coagulating rat PRP in the absence of flow. In recalcified PRP, addition of TF (2 pM) greatly accelerated the generation of thrombin (Fig. 1A). We verified that this thrombin generation was almost completely abolished by preincubation with a saturating dose of heparin (reducing the ETP by >95%). Furthermore, thrombin generation fully relied on activated platelets, because it was abolished when PFP instead of PRP was used (data not shown), and it was fully blocked in the presence of 30 µg mL^{-1} PS-scavenging annexin A5 (peak height 1.6±0.5% of control, Fig. 1B).

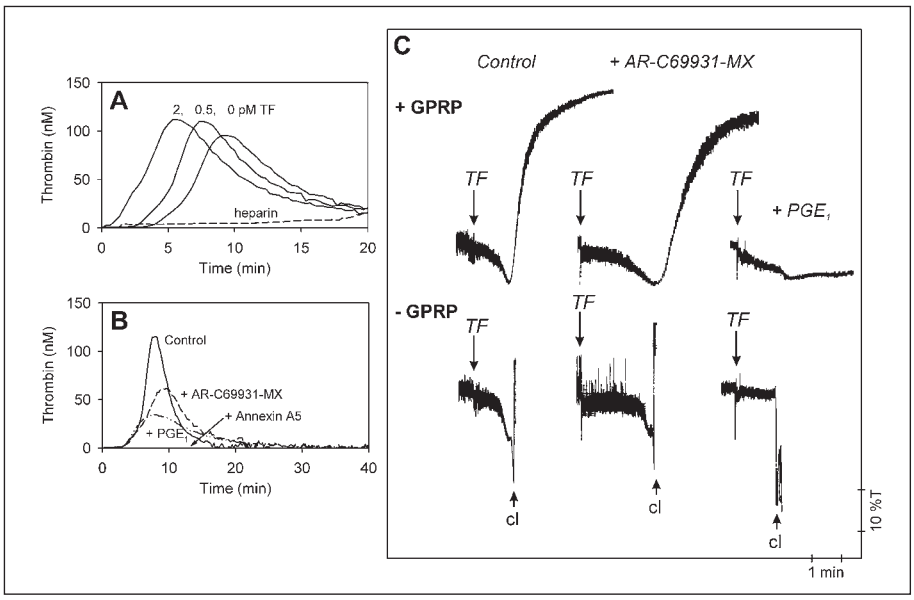

Figure 1: Effect of PGE1 and AR-C69931MX on thrombin generation and aggregation in PRP triggered with tissue factor. (A) Thrombin generation was measured in citrated rat PRP triggered with 0–2 pM TF and 16.7 mM $CaCl_2$. Heparin (0.05 U mL^{-1}) was present, where indicated. (B) Effect of PGE_1 (10 µM), AR-C69931MX (10 µM) or annexin A5 (30 µg mL^{-1}) on thrombin generation with TF (2 pM) and $CaCl_2$. (C) Effect of PGE_1 or AR-C69931MX (each 10 µM) on turbidity change in PRP triggered with TF (2 pM) and $CaCl_2$ under stirring conditions. Experiments were performed in the presence or absence of GPRP (2 mM); cl = formation of clot. Traces are representative for 5–7 experiments.

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Preincubation of rat PRP with 10 μM PGE_1 resulted in greatly reduced, but not abrogated, thrombin formation with a thrombin peak height of 28.0±8.6% (n=5, P<0.05) compared to control (Fig. 1B). Typically, this peak was reached at an earlier time point, i.e. 2.1±0.5 min earlier than in the control condition. Preincubation of rat PRP with 10 μM AR-C69931MX gave a smaller reduction in peak height to 53.9±3.1% (n=5, P<0.05); the peak was now delayed with 3.1±1.0 min compared to control. The effects of PGE_1 and AR-C69931MX did not add up: combined preincubation gave a peak height of 22.0±4.3% of control. Furthermore, the AR-C69931MX effect was not influenced by addition of 20 μM MRS 2179 (an antagonist of the second platelet ADP receptor, $P2Y_1$), giving a peak height of 61.7±3.5% of control. This indicates that, similarly as reported for human platelets (17), the procoagulant effect of ADP is mediated by $P2Y_{12}$ receptors.

The efficacy of AR-C69931MX and PGE_1 in inhibiting platelets under conditions of thrombin generation was evaluated by turbidometry. Triggering of citrated PRP with TF/$CaCl_2$ led to shape change of platelets within 30 s, followed by formation of a clot (Fig. 1C). The clot formation was prevented by the peptide GPRP, which inhibits the polymerization of fibrin. In the presence of GPRP, the shape change in response to TF/$CaCl_2$ was followed by rapid aggregation of platelets. Preincubation with AR-C69931MX delayed both shape change and aggregation, as was observed in the presence of GPRP (Fig. 1C). Preincubation with PGE_1 further delayed shape change and completely inhibited aggregation. Thus, in TF-triggered rat PRP, both compounds had an aggregation-delaying effect in addition to their inhibiting effect on thrombin generation.

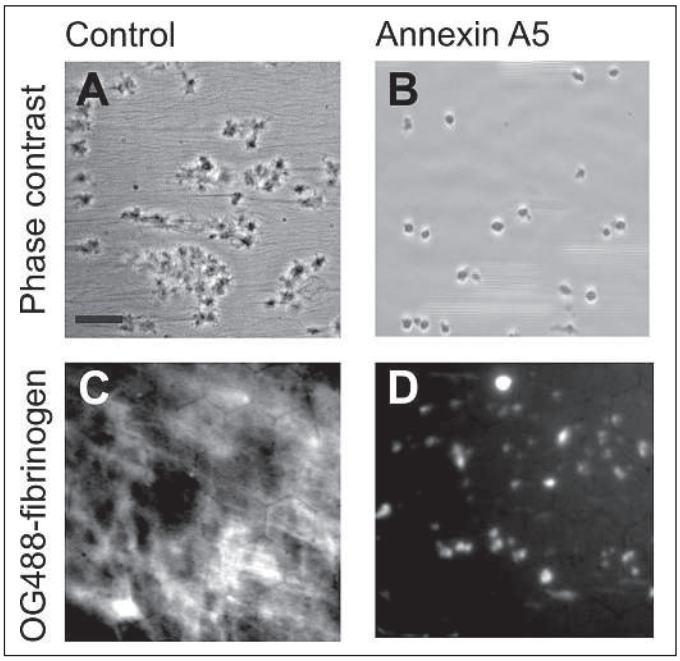

Figure 2: Effect of annexin A5 on thrombus and fibrin formation in flowing blood triggered with tissue factor. Citrated rat blood containing 0.2 mg mL^{-1} OG488-fibrinogen was perfused during 5 min over a fibrinogen surface at a shear rate of 250 s^{-1}. During perfusion coagulation was triggered with 2 pM TF and 16.7 mM $CaCl_2$. The blood were pretreated with a high dose of 30 μg mL^{-1} unlabeled annexin A5, where indicated. Shown are representative phase contrast (A, B) and fluorescent images (C, D), captured after 8 min (n=3). Bar=10 μm.

Effects of platelet inhibitors on thrombus formation and coagulation in rat blood under flow

To study the effects of platelet inhibitors under conditions of flow and coagulation, whole rat blood was triggered with TF/$CaCl_2$ and perfused over a fibrinogen surface at a moderately low wall-shear rate of 250 s^{-1}. Perfusion of blood with $CaCl_2$ plus 2 pM TF resulted in early platelet deposition and formation of fibrin fibers, which started after about 2 min. Platelets later assembled into aggregates, leading to 29.7±2.6% (n=12) of the fibrinogen surface area covered with multi-layered thrombi after 5 min of flow (Fig. 2A). At a more advanced stage (7–8 min), the chamber became occluded by large fibrin-platelet thrombi. Addition to the blood of 30 μg mL^{-1} annexin A5 (chelating exposed PS), completely abolished aggregate formation, while only few platelets adhered (Fig. 2B). That annexin A5 acted as an anticoagulant was confirmed by experiments where OG488-fibrinogen was added. In the control condition, networks of OG488-labeled fibrin and platelets were observed after perfusion (Fig. 2C). With annexin A5 present, fluorescent fibers remained absent and only single platelets displayed fibrinogen labeling (Fig. 2D). This indicated that the formation of aggregates and fibrin during flow relied on PS exposure and subsequent thrombin formation, putatively on activated platelets.

Further flow experiments confirmed that platelet activation and aggregate formation were enhanced by TF. Perfusion of blood without TF resulted in a delayed adhesion of mostly individual platelets. First traces of fibrin fibers did not appear before 5 min of flow (Fig. 3B). To show that the TF-induced process relied on thrombin formation, a saturating dose of heparin was added, which indeed completely abolished platelet deposition and fibrin formation during the experimental time (Fig. 3C). The activation state of adhered platelets was verified by post-staining with OG488-annexin A5 to detect PS-exposing platelets (Fig 3D-F). The few adherent platelets hardly exposed PS in the presence of heparin. When OG488-annexin A5 label was added to the flowing blood (0.5 μg mL^{-1}, a low dose not influencing coagulation), it could be verified that heparin prevented the appearance of PS-exposing platelets during the whole flow experiment (Fig. 3G).

To monitor the changes in activation state of platelets during adhesion, blood was supplemented with 2% Fluo-3 loaded platelets from the same animal. Upon perfusion with TF/$CaCl_2$, adherent fluorescent platelets displayed a prolonged increase in $[Ca^{2+}]_i$, starting at the moment of adhesion or after a short lag-time (Fig. 4A). This pointed to a high activation state of the cells. In the absence of TF, $[Ca^{2+}]_i$ in adherent platelets initially remained low. However, after 5 min of flow, most cells responded by series of spiking rises in $[Ca^{2+}]_i$ (Fig. 4B). Further control experiments indicated that with heparin or annexin A5 present (at concentrations completely blocking thrombin generation), platelets remained low in $[Ca^{2+}]$i except for incidental spikes (Fig. 4C, D). The majority of the Ca^{2+} responses thus were secondary to generation of thrombin. Together, these data indicate that under flow the TF/$CaCl_2$-triggered formation of aggregates and

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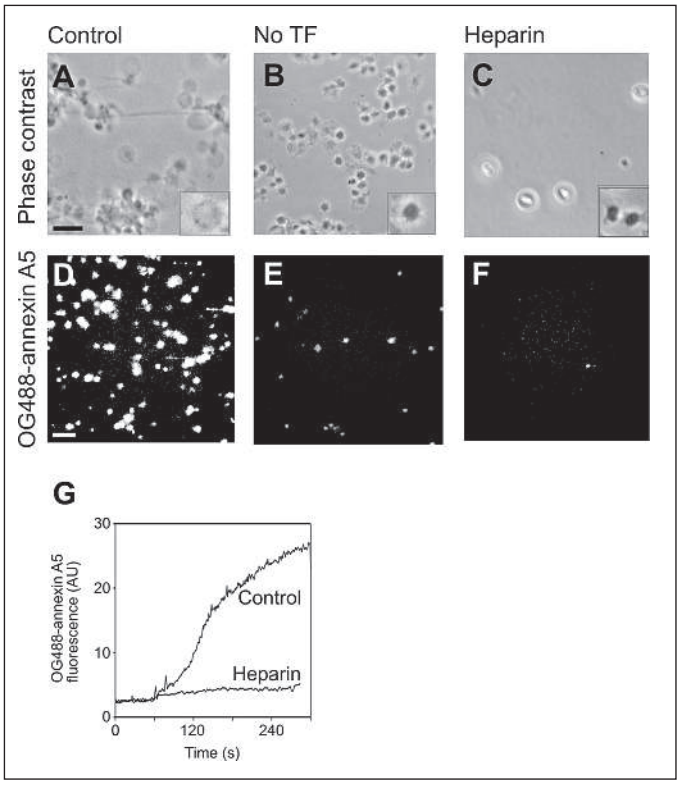

Figure 3: Contribution of thrombin to thrombus formation and PS exposure in tissue factor-triggered, flowing blood. Citrated rat blood was perfused during 5 min over a fibrinogen surface at a shear rate of 250 s^{-1}. During perfusion, coagulation was triggered with 2 pM TF and 16.7 mM $CaCl_2$ (A, D); with 16.7 mM $CaCl_2$ alone (B, E); or with TF/$CaCl_2$ and 0.05 U mL^{-1} heparin (C, F). Representative images are presented, which were taken after 5 min (*n*=4–10 experiments). (A-C) Phase-contrast images; (D-F) fluorescence images after staining post-perfusion with 0.5 µg mL^{-1} OG488-annexin. (G) Accumulation of fluorescence at fibrinogen surface during perfusion of blood, labeled with 0.5 µg mL^{-1} OG488-annexin A5, triggered with TF/$CaCl_2$. Heparin (0.05 U mL^{-1}) was present as indicated. Bar=10 µm; inserts show 2× magnifications.

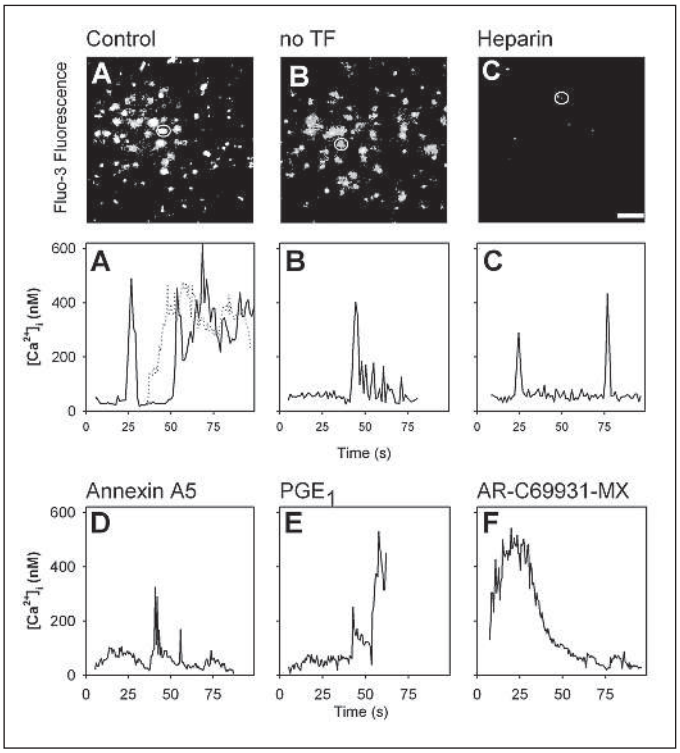

Figure 4: Platelet Ca^{2+} responses in flowing blood triggered with tissue factor. Citrated rat blood containing 2% Fluo-3 loaded platelets from the same animal was triggered with TF/$CaCl_2$ during perfusion (see Fig. 3). Rises in $[Ca^{2+}]_i$ in single adherent platelets were determined after about 3 min of flow. Coagulation was triggered during perfusion with 2 pM TF and 16.7 mM $CaCl_2$. (A) Control condition, (B) triggering without TF. Further, pretreatment of the blood with 0.05 U mL^{-1} heparin (C), 30 µg mL^{-1} annexin A5 (D), 10 µM PGE_1 (E), or 10 µM AR-C69931MX (F). Single-platelet Ca^{2+} responses are representative for those of 16–45 cells.

fibrin is driven by the procoagulant action of activated, PS-exposing platelets and subsequent thrombin generation. Indeed, without coagulation (heparin) or procoagulant platelets (annexin A5), thrombus formation was absent.

Effects of the platelet inhibitors PGE_1 and AR-C69931MX were studied in this flow model in the presence of TF-triggered coagulation. Pretreatment of blood with PGE_1 (10 µM) resulted in deposition of mostly single platelets (Fig. 5A). Fibrin fibers were still formed. In comparison to the control condition, area coverage by platelets was not reduced after 5 min of flow (Fig. 5B). PGE_1 influenced the activation state of adhered platelets: the Ca^{2+} responses showed a consistent lag-time (Fig. 4E) and the surface coverage with PS-exposing platelets was about halved (Fig. 5B). Nevertheless, platelets gradually spread over the surface and some formed blebs, which are morphologic indications for (delayed) activation. When blood was pretreated with AR-C69931MX (10 µM), similar effects were seen as with PGE_1: platelets mostly adhered as single cells, but sometimes assembled into micro-aggregates and fibrin was still formed (Fig. 5A). Furthermore, the AR-C69931MX treatment reduced PS exposure (Fig. 5B) and gave transient Ca^{2+} responses (Fig. 4F). This indicated that both agents effectively suppressed platelet aggregate formation under flow, but only partially inhibited the activation state of the deposited platelets in terms of $[Ca^{2+}]_i$ elevation and PS exposure.

Effects of platelet inhibitors on thrombus formation and coagulation in human blood under flow

To investigate the relevance of these findings for the human system, we performed a similar set of flow experiments with human blood under conditions of coagulation at a shear rate of 250 s^{-1}. The onset of stable platelet adhesion and the formation of platelet aggregates and fibrin fibers was relatively slow when TF/$CaCl_2$-triggered human blood was perfused over fibrinogen in comparison to rat blood. After several min of perfusion, the human platelets adhered and became activated so that, after 6 min, aggregated platelets had covered 21±2% (*n*=3) of the fibrinogen surface (Fig. 6A). After staining for PS exposure with labeled annexin A5, 2.6±0.7% of the surface was covered with fluorescent platelets, which was lower than seen with rat blood. Control experiments showed that heparin addition (at a concentration abrogating thrombin generation), as with rat blood, com-

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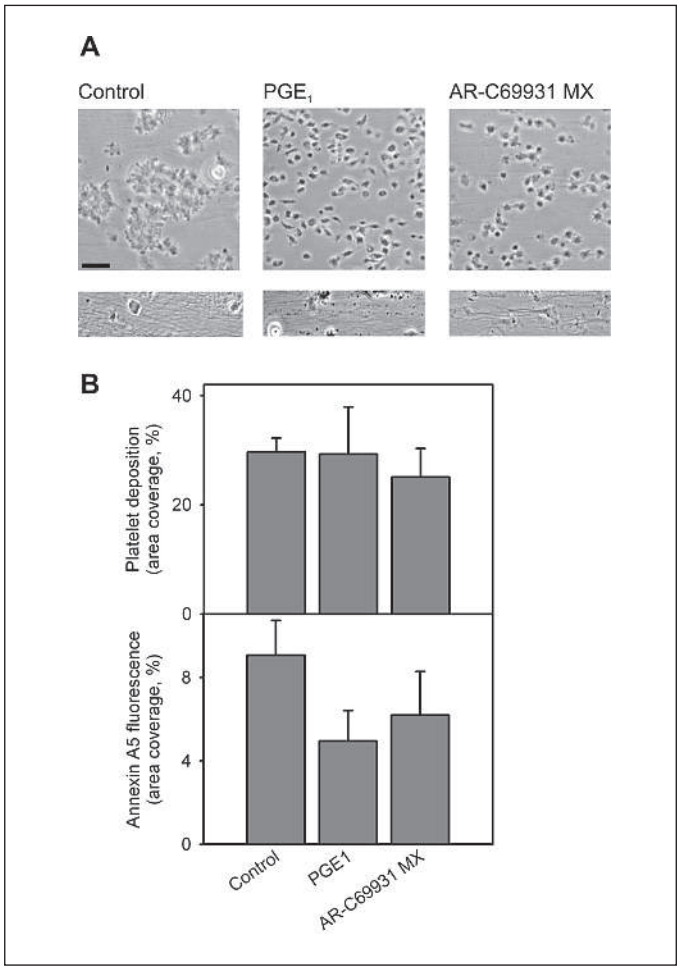

Figure 5: Effects of PGE_1 and AR-C69931MX on thrombus formation and platelet activation in flowing rat blood triggered with tissue factor. Flow experiments were carried out in the presence of $TF/CaCl_2$, as described for Fig. 3. Rat blood was pretreated with PGE_1 or AR-C69931MX, each 10 µM as indicated. (A) Representative phase-contrast images taken after 5 min of perfusion. *Upper panels*: images taken slightly above the coverslip surface; *lower panels*: images of fibrin fibers at the focal plane of the surface (bar=10 µm). (B) Surface area coverage with platelets or OG488-annexin A5 fluorescence after 5 min of perfusion. Mean±SEM (*n*=4–6 experiments).

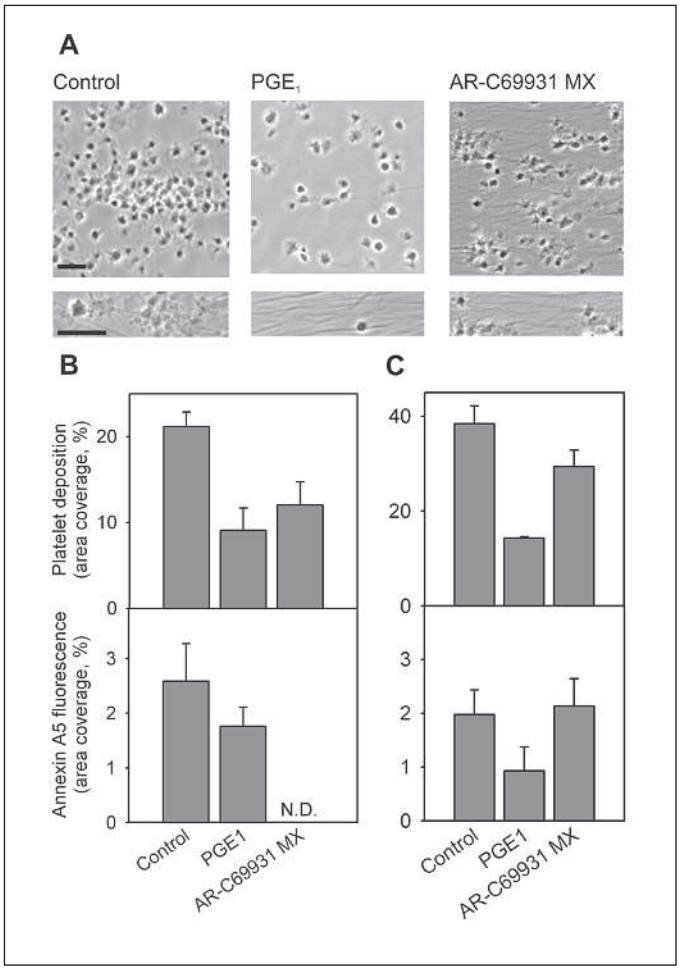

Figure 6: Effects of PGE_1 and AR-C69931MX on thrombus formation and platelet activation in flowing human blood triggered with tissue factor. Flow experiments were carried out in the presence of $TF/CaCl_2$, as indicated for Fig. 3. Human blood was pretreated with 10 µM PGE_1 or 10 µM AR-C69931MX, as indicated. (A) Representative phase-contrast images and (B) surface area coverage after 6 min of perfusion at a shear rate of 250 s^{-1} (N.D., not determined). *Upper panels*: images taken slightly above the coverslip surface; *lower panels*: images of fibrin fibers at the focal plane of the surface (bar=10 µm). Surface area coverage is given with platelets or OG488-annexin A5 fluorescence (stained post-perfusion). (C) surface area coverage after 4 min of perfusion at 1500 s^{-1}. Mean±SEM (*n*=2–3 experiments).

pletely inhibited platelet aggregation and PS exposure as well as fibrin formation; only few single platelets adhered. In the presence of PGE_1 (10 µM), aggregate formation was completely prevented. However, with human blood, many single platelets adhered, which had an activated shape and spread over the surface; fibrin fibers were still formed. Surface coverage of OG488-annexin A5 fluorescence was only partly reduced by 30% compared to the control (Fig. 6B), similar as seen with rat platelets.

When the flow experiments were performed at a higher (arterial) shear rate of 1500 s^{-1}, essentially the same effects of PGE_1 treatment were obtained (Fig. 6C). Inhibition of the ADP receptor $P2Y_{12}$ by AR-C69931MX (10 µM) resulted in similar, but less potent effects: reduced aggregate formation and still fibrin formation and PS exposure. Under static conditions, using TF-triggered, defibrinated human PRP, we found that PGE_1 treatment (10 µM) suppressed integrin αIIbβ3 activation with 83% (platelet binding of FITC-labeled PAC1, an antibody against activated αIIbβ3, as measured by flow cytometry), while thrombin was still formed (peaking at 40 nM). Assuming that PGE_1 has the same effect under flow, we can conclude that a substantial but incomplete suppression of integrin activation can abolish platelet aggregation, but still allow platelet adhesion to fibrinogen. Taken together, these data show that in flowing, coagulating blood from rat and human, either inhibitor suppressed platelet aggregation, but no more than delayed formation of thrombin and fibrin, or PS exposure on activated platelets.

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Discussion

Photochemically-induced thrombus formation is considered to be a sensitive method to detect hypocoagulant conditions in laboratory animals, e.g. as induced by dietary fish oil (27). Surprisingly, using this thrombosis model in rats, we did not find effects of moderate anticoagulant treatment, with either heparin (inactivating factor Xa and thrombin) or warfarin (reducing levels of vitamin K-dependent coagulation factors). Nevertheless, this treatment caused major reduction in thrombin generation, when measured *ex vivo* in tissue factor-triggered PRP. Because these results suggested that the mere down-regulation of coagulant activity is insufficient to reduce thrombosis tendency, we further explored the effects of two antiplatelet agents which, in addition, are known to reduce thrombin generation.

First, PGE_1 was used, which causes EP/IP receptor-mediated, Gs-dependent elevation in cyclic AMP in platelets, resulting in down-regulation of Ca^{2+} responses, inactivation of αIIbβ3 integrin, and inhibition of platelet aggregation (32–34). Second, AR-C69931MX was used to block the $P2Y_{12}$ receptor (21, 22). This ADP receptor, coupled to Gi, contributes to ADP-mediated platelet aggregation as well as thrombin generation (17, 18, 20). In human PRP, the reducing effects of these agents on thrombin generation are due to inhibition of appearance of procoagulant PS at the platelet surface (16). We thus assessed the effects of PGE_1 and AR-C69931MX on procoagulant activity of rat platelets by performing static thrombin generation experiments with PRP and, more physiologically, *ex vivo* flow experiments with coagulating whole blood. In both sets of experiments, the extrinsic coagulation pathway was triggered with TF. Most flow studies were performed at relatively low shear rate (250 s^{-1}), as thrombin is considered to play a prominent role under this condition.

Perfusion of whole rat blood over a fibrinogen surface upon simultaneous triggering with TF/$CaCl_2$ allowed detection of initial and late coagulant effects: platelet adhesion and activation, PS exposure, fibrin formation and platelet aggregation. Adhered platelets showed high, prolonged Ca^{2+} responses that were accompanied by bleb formation and surface exposure of procoagulant PS. These platelet responses were thrombin-mediated, as they were absent in control experiments with heparin. Labeling experiments with OG488-annexin A5 showed a good correlation between the appearance of PS-exposing (blebbing) platelets and fibrin. Blocking experiments with high annexin A5 demonstrated that the exposed PS is critical for the formation of thrombin and fibrin, proving that procoagulant platelets play a key role in this process. The above experiments thereby illustrate that the positive feedback loop, in which initial thrombin formation stimulates platelet aggregate formation and exposure of coagulation-stimulating PS, is also operative under conditions of flow.

We note that in the initial *in vivo* experiments heparin and warfarin were used at moderate concentrations to partially suppress thrombin generation. On the other hand, in the flow experiments with whole blood, heparin was applied *in vitro* at maximally effective dose, sufficient to completely block thrombin/fibrin formation, to verify that the observed platelet responses were thrombin-dependent. However, as *in vivo* also the vessel wall plays a regulatory role in thrombus formation, the apparent inability of anticoagulants to influence *in vivo* thrombosis can also be due to the presence of activating substances at the (damaged) vessel wall.

The flow results obtained with platelet inhibitors under conditions of TF-triggered coagulation were unexpected. In both rat and human blood, PGE_1 prevents the formation of platelet aggregates in flowing blood (Figs. 5–6), even until occlusion of the flow chamber by massive fibrin deposition. AR-C69931MX has a similar, though more moderate effect. In agreement with the notion that PGE_1 and AR-C69931MX considerably but incompletely inhibit PS-dependent thrombin generation under stasis in PRP from rat (Fig. 1B) and human (16), these agents also only partially reduce PS exposure and fibrin formation in flowing blood (Fig. 6). Consistent with these results, other authors have described that PGE_1 delays fibrin clot formation in TF-triggered whole blood under static conditions (36). Overall, we can conclude that PGE_1 and AR-C69931MX inhibit the various aspects of coagulation-induced platelet activation in a different way. They extensively reduce integrin activation, but only delay Ca^{2+} signaling and PS exposure in response of the thrombin that is formed upon coagulation. That platelets deposited from PGE_1– and AR-C69931MX-treated blood are in an activated state is also apparent from their changes in morphology, i.e. spreading over the surface and forming blebs. The final result is abolished (with PGE_1) or suppressed (with AR-C69931MX) platelet aggregation, yet leaving sufficient PS exposure on activated platelets to maintain platelet-dependent coagulation (thrombin and clot formation). Both rat and human platelets behave similarly in the presence of PGE_1 or AR-C69931MX under coagulant conditions. Yet, subtle differences are observed between the rat and human systems, as in human blood deposition of platelets is reduced in the presence of PGE_1 or AR-C69931MX, while in rat blood this remains unchanged. This can be explained, for instance, by the higher platelet count of rat blood and different levels of adhesive receptors on the platelets from this species.

The present results thus indicate that platelet aggregation is more sensitive to platelet inhibitors such as PGE_1 than PS exposure and subsequent formation of thrombin and fibrin. In agreement with this, others have observed that prostaglandin-induced down-regulation of integrin αIIbβ3 activation is of longer duration than the platelet Ca^{2+} signal (34). Our findings, using whole blood in which coagulation is triggered with TF/$CaCl_2$ under flow conditions, are a clear indication that this differential regulation of platelet responses is of physiological relevance.

Interestingly, for the human system, we find that treatment of TF-triggered blood with PGE_1 gives reduced platelet aggregation with only partial reduction of PS exposure upon perfusions at low and high shear rates of 250 and 1500 s^{-1}, respectively. This finding is of potential clinical interest. For many years, patients with peripheral arterial obstructive disease are being treated by intravenous administration of PGE_1 (alprostadil). The mechanism for the therapeutic efficacy is not fully clear yet; moderate increases in blood flow have been measured as well as reduced expression of endothelial activation markers and increased production of pro-angiogenic factors (37–39). The present results suggest that part of the beneficial effect of alprostadil can lie in a reduction of aggregate and thrombus formation under conditions where coagulation is still sufficiently active. If

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this proves to be true, it argues for the use of inhibitors which suppress both platelets and thrombin generation as antithrombotic agents. These positive effects of substances with anti-aggregatory as well as anti-coagulatory properties thus need to be taken further in future human studies.

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