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Nitridergic Platelet Pathway Activation by Hementerin, a Metalloprotease from the Leech *Haementeria depressa*

Ana M. Chudzinski-Tavassi[1,a,*], Emilse Bermejo[2,a], Ruth E. Rosenstein[3], Fernanda Faria[1], María I. Keller Sarmiento[3], Fabiana Alberto[2], Misako U. Sampaio[4] and María A. Lazzari[2]

[1] Laboratório de Bioquímica e Biofísica, Instituto Butantan, Av. Vital Brazil 1500, CEP 05503-900, São Paulo, Brazil
[2] IIHEMA, Academia Nacional de Medicina de Buenos Aires, Buenos Aires, Argentina
[3] Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[4] Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, Brazil

*Corresponding author

Hementerin (HT) is an 80 kDa fibrino(geno)lytic metalloprotease, purified from saliva of the leech *Haementeria depressa*. In the present report, the effect of HT on several functional parameters of human platelets was assessed. HT inhibited platelet aggregation and ATP release induced by different agonists such as ADP, adrenaline, collagen, thrombin, and arachidonic acid. HT did neither modify the expression of platelet glycoproteins (Ib, IIb-IIIa, Ia-IIa, IV) nor intraplatelet fibrinogen levels, whereas it markedly decreased CD62P and CD63 levels after the stimulation with thrombin. HT significantly increased thrombin-induced platelet Ca^{2+} intracellular levels, cGMP content and nitric oxide synthase (NOS) activity. The effect of HT on platelet aggregation was reversed by two NOS inhibitors, N^{ω}-Nitro-L-arginine methyl ester and 2 N^{G}-Nitro-L-arginine. In summary, these results indicate that HT is an effective inhibitor of human platelet aggregation, presumably through activation of the platelet's nitridergic pathway.

Key words: Coagulation / Fibrinogenolysis / Nitric oxide.

Introduction

Blood-sucking animals possess salivary substances that keep blood from clotting and/or can degrade blood proteins (Arocha-Pinango *et al.*, 1999; Basanova *et al.*, 2002). In spite of the diversity of blood-sucking species, all compounds secreted by these animals that affect host hemostasis can be subdivided into the following groups: i) regulators of fibrin formation, comprising inhibitors of thrombin (Thr) and factor XIIIa, fibrino(geno)lytic enzymes and activators of fibrinolysis, ii) inhibitors of activation of intrinsic mechanisms of blood coagulation and proteins of the prothrombinase complex, and iii) inhibitors of platelet function (Arocha-Pinango *et al.*, 1999; Basanova *et al.*, 2002). All these molecules occur in different leech genera. Hirudin, described in 1884, was the first anticoagulant characterized from the salivary glands of the European leech *Hirudo medicinalis* and has been studied in detail (Haycraft, 1884). Hirudin is a potent inhibitor of thrombin (Markwardt, 1991) and its recombinant form obtained in *E. coli* by different groups (Bergman *et al.*, 1986; Harvey *et al.*, 1986) is actually being used in patient therapy (Shen, 2001; Weitz *et al.*, 2003). Similar active proteins have been identified in leeches from the genus *Haementeria*, such as hementerin and lefaxin from *Haementeria depressa* (Chudzinski-Tavassi *et al.*, 1998; Faria *et al.*, 1999), hementin and ghilianten from the giant Amazon leech *Haementeria ghilianii* (Budzynski *et al.*, 1981) and antistasin from *Haementeria officinalis* (Tuszynski *et al.*, 1987).

Platelet aggregation inhibitors from the salivary gland secretion of *Hirudo medicinallis* involve several molecules including hirudin, apyrase, platelet activating factor (PAF) inhibitor, an adenylate cyclase activator, a platelet adhesion and aggregation inhibitor (calin), and an inhibitor of spontaneous platelet aggregation (destabilase; Basanova *et al.*, 2002). LAPP (leech anti platelet protein) is a protein isolated from the soluble fraction of whole extract of *Haementeria officinalis*. It selectively inhibits collagen-induced platelet aggregation (Connolly *et al.*, 1992). Potent glycoprotein IIb-IIIa antagonists, as well as platelet aggregation inhibitors, were isolated from *Macrobdela decora* (decorsin; Seymour *et al.*, 1990) and *Placobdella ornata* (ornatins; Mazur *et al.*, 1991) leeches.

We have purified and described the terminal sequence of hementerin (HT), a protein contained in crude extracts of the salivary complex of the *Haementeria depressa* leech, (Chudzinski-Tavassi *et al.*, 1998). It is a single chain 80 kDa, $PhMeSO_2F$-resistant, calcium-dependent metalloproteinase, which specifically degrades fibrin(ogen) through a plasminogen-independent pathway.

In the present report, we have studied the effect of HT on several parameters of platelet function and we present evidence supporting the theory that HT inhibits platelet reactivity by increasing nitric oxide (NO) production.

[a] These authors contributed equally to this work.

Results

Figure 1A depicts the effect of HT on platelet aggregation induced by collagen. 90, 75, 47.5, and 25% platelet aggregation was observed in the presence of 2.5, 5, 7.5, and 10 μg/ml HT, respectively, as compared to control conditions, with the IC50 being about 7.5 μg/ml. In another set of experiments, platelet-rich plasma (PRP) was incubated with buffer or HT (7.5 μg/ml) at 37 °C during 0, 1, 2, or 15 min before activation with collagen (1 μg/ml) as shown in Figure 1B. The inhibitory effect of HT on platelet aggregation was already evident at 2 min of incubation. After this period of incubation with HT, no changes on fibrinogen concentration were observed, whereas at 15 min a decrease of 20% in this parameter was detected. Fibrinogen degradation products (PDFs) were not detected at either examined interval.

To further examine the effect of HT on platelet aggregation, several agonists were assayed. In the case of thrombin, washed platelets (WPs) were used (Figure 2). HT significantly inhibited platelet aggregation induced by Thr, ADP, adrenaline (ADR), and arachidonic acid (AA).

ATP release triggered by both ADR and collagen was completely inhibited after 2 min of preincubation with 7.5 μg/ml HT, as shown in Figure 3.

In order to evaluate whether HT modulates platelet glycoprotein expression, in the next experiments we performed flow cytometry studies. As shown in Table 1, there were no significant differences in the mean fluorescence intensity of CD 42b (Ib), CD41 (IIb) CD 61 (IIIa) CD29 (Ia) or CD36 (GP IV) between PRP incubated with Tyrode buffer or HT for 0, 2, and 15 min. Table 2 summarizes the effect of HT on CD62p and CD63 levels in WPs after the stimulation with thrombin. HT significantly inhibited the expression of these activation markers under these conditions. Isotypic controls (FITC) did not change after the stimulation with thrombin in the presence or absence of HT (data not shown).

To gain insight into the mechanism of action of HT, WP, intracellular Ca^{2+} levels in the presence of thrombin alone or in combination with HT were assessed. In our experimental conditions, thrombin *per se* augmented this parameter, but its combination with HT provoked a further increase in human platelet Ca^{2+} levels (Figure 4).

Figure 5 shows the effect of HT on cGMP accumulation in the presence of 3-isobutyl-1-methylxanthine (IBMX) in WPs stimulated with Thr. HT significantly increased thrombin-induced cGMP accumulation although none of these compounds showed any effect when incubated alone.

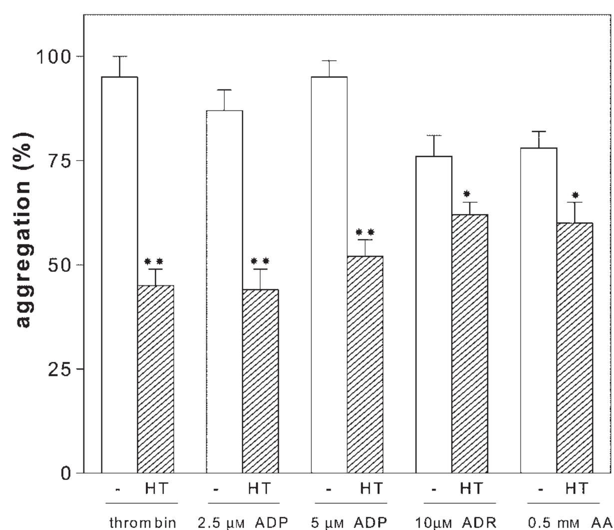

Fig. 2 Effect of HT on Platelet Aggregation Induced by Several Agonists.
In the case of thrombin, WPs were used. PRP or WPs were preincubated with or without HT (7.5 μg/ml) for 2 min, then Thr (0.5 U/ml), ADP (2.5 and 5 μM), ADR (10 μM) or AA (0.5 mM) were added. Data are the mean values ± SEM of three independent experiments, performed in triplicate. $*p < 0.05$, $**p < 0.01$, by Student's t-test.

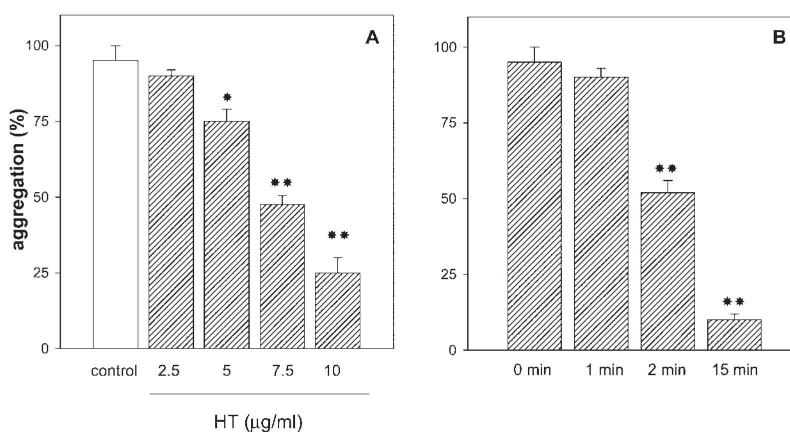

Fig. 1 Effect of HT on Human Platelet Aggregation Induced by Collagen.
(A) PRP was preincubated for 2 min with different concentrations of HT, then 1 μg/ml collagen was added. (B) Time course of HT effect on platelet aggregation. Data are the mean values ± SEM of three independent experiments performed in triplicate. $*p < 0.05$, $**p < 0.01$, by Dunnett's test.

The effect of HT on nitric oxide synthase (NOS) activity was examined in WPs. As shown in Figure 6, neither HT nor thrombin *per se* affected NOS activity, while the combination of both compounds significantly increased the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. The effect of HT was already evident after 5 min of incubation and it did not augment when the incubation was extended to 15 min. A Ca^{2+} ionophore (A23187) also increased this enzymatic activity. The effect of HT on platelet aggregation induced by thrombin was blocked by the incubation in the presence of two NOS inhibitors, N^{ω}-Nitro-L-arginine methyl ester (L-NAME; Figure 7), and 2 N^G-Nitro-L-arginine (data not shown).

Table 1 Measurement of Platelet-Bound Antigens on PRP by Flow Cytometry.

Antibodies	Tyrode	Hementerin Time (min)		
		0	2	15
CD42b	90±2	92±1	90±3	85±3
CD41	105±2	104±1	100±1	98±2
CD61	114±4	110±2	108±3	100±3
CD29	56±1	53±2	51±1	50±2
CD36	98±1	97±2	98±1	99±2

Hementerin at 7.5 µg/ml: values represent the mean fluorescence intensity (MFI) ± SD (n=3). HT did not significantly affect the levels of any of these proteins.

Table 2 Measurement of Activation Markers on WPs by Flow Cytometry.

Antibodies	WPs		
	Tyrode	Tyrode + Thr	Tyrode + Thr + HT
CD62	6±1	52±3[a]	17±1[b]
CD63	6±1	58±2[a]	14±4[b]

WPs: washed platelets, Thr: thrombin 0.5 U/ml, HT: hementerin 7.5 µg/ml, MFI: mean fluorescence intensity. Values represent the MFI ± SD (n=3). [a]$p < 0.01$ *vs.* control, [b]$p < 0.01$ *vs.* Thr, by Tukey's test.

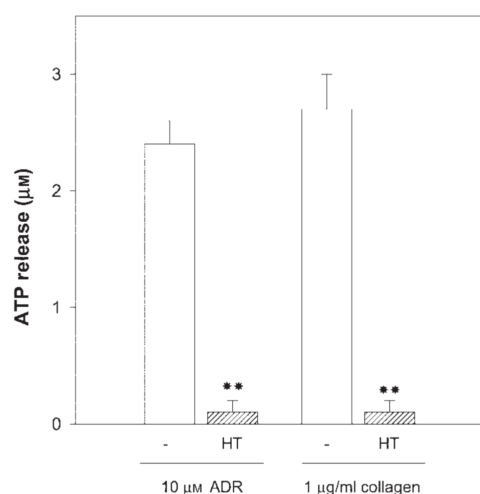

Fig. 3 Effect of HT on ATP Release Induced by ADR or Collagen.
PRP was preincubated with or without HT (7.5 µg/ml) for 2 min. Then, ATP release was triggered by ADR (10 µM) or collagen (1 µg/ml). HT significantly blocked this parameter. Data are the mean values ± SEM of three independent experiments, performed in triplicate. **$p < 0.01$, by Student's t-test.

Discussion

The results reported here demonstrate that HT, a metalloprotease, effectively inhibited human platelet aggregation in a rapid and concentration-dependent manner. In platelets preincubated with HT a complete inhibition of

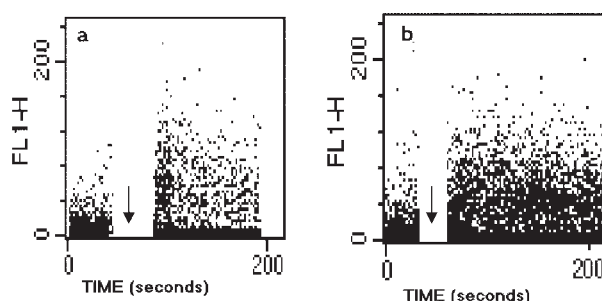

Fig. 4 Effect of HT on Intracellular Calcium Levels.
WPs were preincubated with or without HT (7.5 µg/ml) for 2 min. (A) Effect of 0.5 U/ml thrombin in the absence of HT. (B) Effect of 0.5 U/ml thrombin in the presence of HT. Arrows indicate the addition of thrombin. Data were analyzed as described in Materials and Methods.

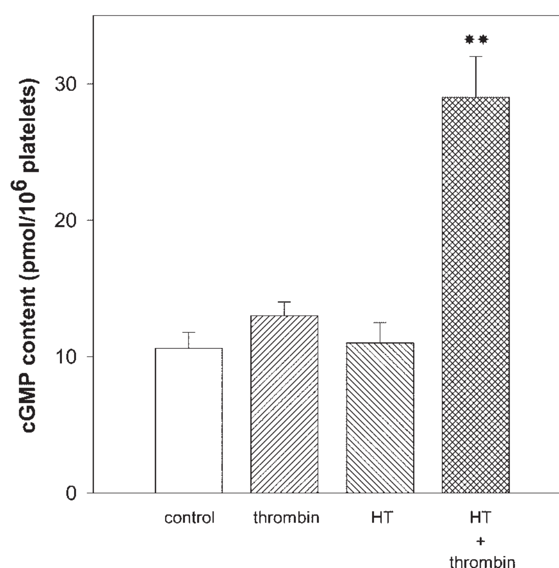

Fig. 5 Effect of HT on Platelet cGMP Accumulation.
WPs were preincubated with IBMX (0.5 mM) in the presence or absence of 7.5 µg/ml HT and further incubated with or without thrombin for 5 min. Data are the mean values ± SEM of three independent experiments, performed in quadruplicate. **$p < 0.01$, by Dunnett's test.

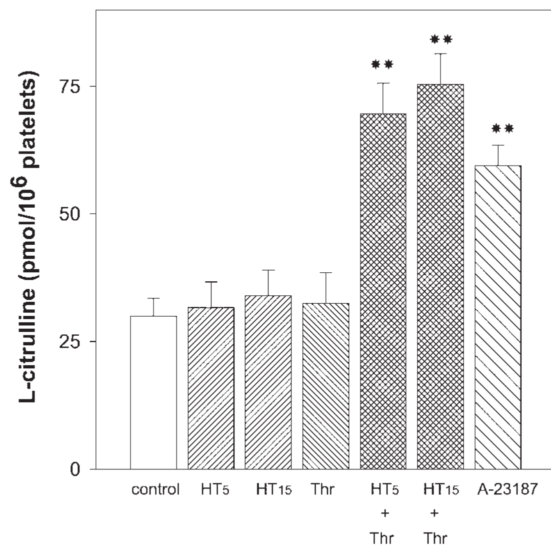

Fig. 6 Effect of HT on Human Platelet NOS Activity.
WPs were preincubated in the presence or absence of HT (7.5 µg/ml) during 5 min (noted as HT5) or 15 min (noted as HT15), and then further incubated with or without 0.5 U/ml thrombin (noted as Thr) for 5 min. NOS activity was assessed through the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as described in Materials and Methods. Data are the mean values ± SEM of three independent experiments, performed in triplicate. $^{**}p < 0.01$, by Dunnett's test.

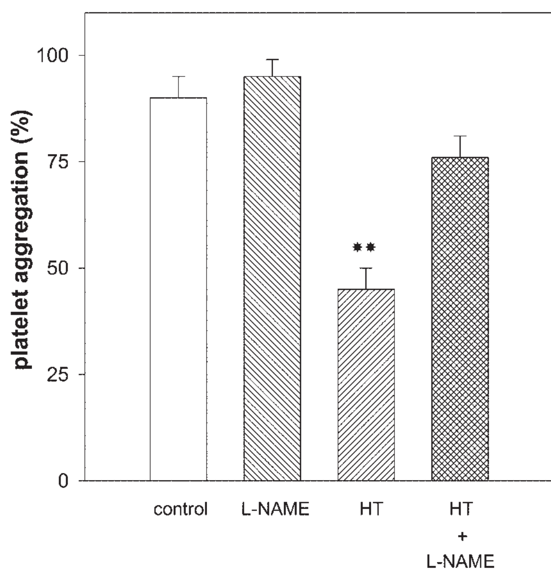

Fig. 7 Effect of L-NAME on the Inhibition Induced by 7.5 µg/ml HT on Platelet Aggregation Stimulated by Thrombin.
WPs were preincubated in the presence or absence of HT with or without 300 µM L-NAME for 2 min, then 0.5 U/ml thrombin were added to all experimental groups. Data are the mean values ± SEM of three independent experiments, performed in triplicate. $^{**}p < 0.01$, by Dunnett's test.

ATP release triggered by ADR and collagen was obtained. Furthermore, the activation markers (CD62P and CD63) significantly decreased when platelets were incubated with HT post-stimulation with thrombin. The inhibition induced by HT on platelet aggregation was evident not only on collagen but also with ADP, ADR, AA, and Thr. These results suggest that HT triggers a general pathway of inhibition, independently of the receptor involved in platelet response. They are different from those described for other leech proteins, including hementin, that inhibit platelet aggregation by their fibrinogenolytic activity (Connolly *et al.*, 1992). Since HT is a fibrino(geno)lytic metalloprotease (Chudzinski-Tavassi *et al.*, 1998) that degrades fibrinogen and fibrin in a plasminogen activation-independent way, its effect on platelet aggregation could be mediated by the proteolysis of fibrinogen. However, several lines of evidence suggest that this is not the case. On the one hand, the inhibitory effect of HT on platelet aggregation was still evident with WPs, without fibrinogen. On the other hand, neither changes in plasmatic fibrinogen nor PDF fragments were observed in the presence of HT.

Since all the agonists used in this study trigger platelet aggregation through Ca^{2+}-dependent mechanisms (Brass, 1984; Benz *et al.*, 1999; Loscalzo, 2001), the effect of HT on intraplatelet Ca^{2+} levels was examined. Paradoxically, although HT did not change Ca^{2+} flux *per se*, it significantly increased the response induced by thrombin. Aside from its role in platelet aggregation, Ca^{2+} is also a well-known activator of endothelial NOS (eNOS) activity (Alderton *et al.*, 2001; Loscalzo, 2001), one of the enzymes responsible for NO generation in both platelets and endothelial cells (Alderton *et al.*, 2001). It is well known that NO derived from eNOS inhibits human platelet function (Moncada *et al.*, 1988; Radomski *et al.*, 1990). Taken together, this evidence prompted us to examine the possibility that NO could be involved in the response induced by HT. This hypothesis was supported by several observations: (i) in the presence of thrombin, HT increased the accumulation of cGMP, the most conspicuous NO second messenger, (ii) NOS activity significantly increased in the presence of HT plus thrombin, and finally, (iii) the effect of HT on platelet aggregation induced by thrombin was blocked in the presence of two NOS inhibitors.

Why the increase in NOS activity and cGMP levels was only evident in the presence of HT and thrombin is not completely understood. As mentioned before, when platelets were incubated with HT and thrombin, the increase of intracellular calcium levels was even higher than that observed in the presence of 0.5 U/ml thrombin alone. Thus, it seems possible that NOS activation requires a threshold of calcium levels that may only be reached in the presence of both drugs. In fact, a similar increase in NOS activity was observed in platelets incubated with ionophore A23187. We can therefore postulate the following sequence in the signaling pathway triggered by HT: (i) an increase in Ca^{2+} flux, (ii) an increase in platelet NOS activity, (iii) cGMP level augmentation, and (iv) inhibition of platelet aggregation through a cGMP-dependent mechanism. This way, when *Haementeria depressa* sucks blood, HT would be injected from the sali-

vary glands into the blood vessels of host animal, and through this mechanism it induces an increase in platelet NO levels that serves as a vasodilator and a platelet aggregation inhibitor to assist blood feeding. An antiplatelet effect related to NO was previously described in the hematophagous insect *Rhodnius prolixus*, whose saliva contains a nitrosyl-heme protein that releases NO (Ribeiro and Walker, 1994). The possibility that other signaling pathways could also be involved in the effect of HT cannot be ruled out at present. In addition, since this signaling pathway was demonstrated only in WPs stimulated with thrombin, the mechanism of action of HT in the presence of the other agonists deserves further examination.

The clinical relevance of platelet-derived NO in patients with acute coronary syndromes was examined (Freedman *et al.*, 1998). In this sense, it has been suggested that an impairment of platelet-derived NO production may contribute to the pathophysiology of this class of cardiovascular diseases (Loscalzo, 2001). Since platelet recruitment is a primary step of thrombus propagation, it is tempting to speculate that an increase in NO production and/or in NO bioactivity could decrease cardiovascular risks. So far, the most common therapeutic strategies aimed to increase NO levels include the use of L-arginine or different NO-releasing donors (Bode-Böger *et al.*, 1996; Kaposzta *et al.*, 2002). Drugs that directly modulate NOS activity or NO bioavailability are less known. It has been described that α-tocopherol enhances platelet and endothelial-derived NO release through antioxidant and PKC dependent mechanisms (Freedman *et al.*, 2000). Antioxidant properties of garlic extracts and its major component S-allyl cysteine were shown to increase bioavailability of NO without changes in eNOS expression and activity (Kim *et al.*, 2001). The antithrombotic and stroke protective effect of statins are mediated in part by eNOS regulation, independently of serum cholesterol levels (Laufs *et al.*, 2000). Here, we presented evidence that the metalloprotease HT raises NO production by increasing platelet NOS activity. Whether such findings are also supposed to extend to endothelial-derived NO remains to be established. Despite the wide range of therapeutic options available for the whole spectrum of ischaemic events, a need for more efficacious and safe compounds still remains. HT is an effective fibrinogenolytic agent with platelet aggregation inhibitory properties and its potential as a therapeutic agent remains to be elucidated.

Materials and Methods

Materials

Hementerin was purified as previously described (Chudzinski-Tavassi *et al.*, 1998) from a crude extract of salivary complexes of the leech, *Haementeria depressa*, by Sephadex G150, an ion exchange column, and by a preparative electrophoresis.

Thrombin and human fibrinogen were obtained from Biopool (Ventura, CA, USA); adrenaline was purchased from Diagnostica (Stago, France), while collagen was obtained from Nycomed (Munich, Germany). Monoclonal antibodies (MoAb; CD42b-PE, CD41-FITC, CD61-FITC, CD29-FITC, CD36-FITC, CD36-FITC, CD62P-FITC, CD63-FITC) and isotype control came from Immunotech (Marseille, France). ADP, ATP, luciferin-luciferase, arachidonic acid, 3-isobutyl-1-methylxanthine, N^{ω}-nitro-L-arginine methyl ester, and 2 N^{G}-nitro-L-arginine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Sample Preparation

Platelet-rich plasma: venous blood was collected from healthy donors who had not taken any medication for one week prior to sampling. Blood samples were collected by venipuncture directly into plastic tubes containing 3.8% sodium citrate (9:1) and centrifuged at 300 *g* for 10 min. PRP was removed and the remainder centrifuged at 1300 *g* for 15 min to obtain platelet-poor plasma (PPP). PRP was adjusted to 300×10^9 l^{-1} using PPP.

Washed platelets: PRP was washed twice in modified Tyrode buffer (138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 6.5) with 50 ng/ml prostaglandin E1. WPs were finally suspended in Tyrode buffer, pH 7.3 with 1 mM $CaCl_2$.

Functional Studies

Platelet aggregation in PRP or WPs was studied by using a chrono-log Lumi aggregometer. PRP samples were incubated with Tyrode or different HT concentrations at 37 °C for 2 min. After incubation, several agonists induced platelet aggregation. In the case of thrombin (0.5 U/ml), WPs were used and HT was analyzed in a similar manner to that with PRP. ATP release was measured simultaneously with aggregation by addition of luciferin-luciferase and the released ATP was calculated from a standard ATP curve.

Flow Cytometry

Flow cytometry was performed with a FACSCalibur (Becton-Dickinson, San Jose, CA, USA) flow cytometer. Light scattering and fluorescence channels were set at logarithmic gain, and at least 10 000 events were collected for each sample. To investigate the effect of HT on constitutive platelet glycoprotein expression, PRP was incubated with or without HT for 0, 2, and 15 min; then 5 μl of these samples were added to tubes containing 50 μl of phosphate-buffered saline (PBS) with saturating concentrations of different MoAbs and collagen or thrombin when activation markers were analyzed. After 40 min incubation at room temperature in the dark, samples were diluted by addition of 450 μl PBS buffer and analyzed. An isotype-matched non-specific MoAb was used as negative control. Changes in the expression of platelet membrane markers were analyzed using the mean fluorescence intensity (MFI).

Assessment of Fibrinogen Levels

A pool of normal plasma was incubated with HT; fibrinogen levels and PDFs were measured by the Clauss clotting time and the latex agglutination method, respectively (Stago, France).

Intracellular Calcium Assessment

Changes in intracellular calcium concentration were monitored using fluo-3/AM, as previously described by Gamberale *et al.* (1998). Briefly, WPs (300×10^9 l^{-1}) were incubated with 4 μM fluo-3/AM for 30 min at 30 °C. Subsequently, loaded cells were washed twice and incubated with 1 mM $CaCl_2$ warmed at 37 °C.

Samples were preincubated with HT or buffer for 2 min, immediately loaded onto the flow cytometer, and basal fluorescence (FL1) was recorded during a 30 s period, after which cells were stimulated with 0.5 U/ml thrombin. The fluorescence intensity was recorded during an additional 200 s interval. For time-dependent measurements, the instrument was set to acquire data continuously over a 200 s period instead of collecting a certain number of events (25 000).

Assessment of Cyclic GMP Levels

Platelets were incubated in 500 µl of buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4 with 0.5 mM IBMX during 30 min and then treated or not during 2 min with HT. After centrifuging for 10 min at 900 *g*, pellets were resuspended in distilled water, boiled for 2 min, and centrifuged at 5000 *g* for 5 min at 4 °C. Cyclic GMP content was measured in the supernatants by RIA after acetylation with acetic anhydride/triethylamine as previously described (Faillace *et al.*, 1995). The acetylated samples and the standard curve were mixed with [^{125}I]-cyclic GMP (15 000 – 20 000 cpm, specific activity 140 mCi/mmol) and a rabbit antiserum (Chemicon, CA, USA) diluted 1:150 and incubated overnight at 4 °C. The antibody complex was precipitated with ethanol at 4 °C using 2% BSA, centrifuged at 2000 *g* for 30 min, and separated by supernatant aspiration. The radioactivity was measured in a gamma counter. The range of the standard curves was 10 – 5000 fmol of cGMP.

NOS Activity Assessment

Human platelet NOS activity was assayed by monitoring the conversion of radiolabelled arginine to citrulline as described (Llomovatte *et al.*, 1997). Briefly, 10^7 cells were incubated in 400 µl 20 mM HEPES, 0.25 M sucrose, and 2.5 mM $CaCl_2$ buffer for 5 min in the presence or absence of HT. Following thrombin or vehicle addition, platelets were further incubated for 5 min and centrifuged at 900 *g* for 10 min. Pellets were resuspended in 50 µl of the same buffer and 50 µl of buffer stock (10 mM HEPES, 2 mM $CaCl_2$, 0.4 mM NADPH, 2 µM FAD, L-[^{3}H]-arginine (5 µCi/ml, specific activity 53.4 Ci/mmol) and 1 µM L-arginine) were added. After incubating for 60 min at 37 °C, the reaction was stopped by adding 200 µl of buffer containing 50 mM HEPES, 10 mM EDTA and 10 mM EGTA (pH 5.5), and centrifuged at 1300 *g* for 10 min. The pellets were disrupted by adding 1 ml of 0.3 M $HClO_4$, neutralizing with 65 µl of 3 M K_2CO_3, and centrifuging at 1300 *g* for 10 min. Aliquots of the supernatant (100 µl) were mixed with 600 µl of Dowex AG50W-X8 (Na^+ form) resin to remove arginine, and centrifuged at 10 000 *g* for 5 min. L-[^{3}H]-citrulline in the supernatant was quantified by liquid scintillation counting. Non-enzymatic conversion of [^{3}H]-arginine to [^{3}H]-citrulline was tested by adding buffer or a heat-inactivated enzyme solution instead of the enzyme source.

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