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Effects of Lopap on Human Endothelial Cells and Platelets

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Key Words

Lopap · Prothrombin activator · *Lonomia obliqua* · Platelets · ICAM-1 · E-selectin · Endothelial cells

Abstract

Severe consumption coagulopathy has been detected in rats after Lopap (a prothrombin activator from *Lonomia obliqua* caterpillar bristles) infusion and in humans after accidental contact with *L. obliqua* bristles. However, platelet count and antithrombin (AT) levels were only modestly affected, suggesting that a different form of blood coagulation activation may be involved in this hemorrhagic syndrome. Here we describe that Lopap had no effect on aggregation of washed human platelets induced by several agonists, suggesting that it might not impair platelet function in vivo. AT was able to inhibit the amidolytic activity of thrombin generated by incubation of Lopap with prothrombin in a purified system, which may be different from that generated by the prothrombinase complex in vivo. The surface expression of both ICAM-1 and E-selectin but not of VCAM-1 was upregulated by Lopap in cultured HUVEC, suggesting that it may behave differently from other mediators, such as thrombin and tumor necrosis factor-α.

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Introduction

Lopap (*Lonomia obliqua* prothrombin activator protease) is a prothrombin-activating serine protease of 69 kD isolated from the crude extract of *Lonomia obliqua* bristles [1, 2]. Lopap showed procoagulant activity since it has been able to shorten the recalcification time of plasma. Infusion of the purified protein provoked thrombus formation in the cremaster microcirculatory network in rats, followed by hemorrhage in ischemic areas. Plas-

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ma obtained from these animals was unclottable whereas platelet count decreased moderately [3].

Patients who had experienced accidental contact with the bristles of *L. obliqua* developed a hemorrhagic diathesis characterized by intense fibrinogen depletion and consumption of coagulation factors, such as factors V, VIII and XIII. Thrombin generation was demonstrated in these patients with the production of fragment 1 + 2 and thrombin-antithrombin complexes. Fibrinolysis was also activated, as shown by extremely high D dimer levels. However, neither a significant fall in platelet count nor a significant reduction in antithrombin (AT) levels was observed in humans, suggesting that a different form of blood coagulation activation may be involved in this hemorrhagic syndrome [4, 5].

A prothrombin activator, such as Lopap, may be involved in the activation of the coagulation cascade in these patients. However, its direct effects on prothrombin may not be the only mechanism by which it causes the bleeding disorder in humans.

In order to understand the possible mechanisms involved in this process, we performed experiments to evaluate: (1) the action of Lopap on aggregation of washed human platelets induced by several agonists; (2) the effect of purified AT on the amidolytic activity of thrombin generated by incubation of Lopap with prothrombin, and (3) the effect of Lopap on the expression of cell adhesion molecules as well as on the production of von Willebrand factor (vWF) by cultured endothelial cells.

Materials and Methods

Materials

RPMI culture medium and fetal bovine serum (FBS) were from Gibco BRL, Grand Island, N.Y., USA. Endothelial cell growth supplement from bovine neural tissue, heparin, pyruvate, adenosine 5′-diphosphate (ADP), α-thrombin, human prothrombin, AT and prostaglandin E_1 (PGE_1) were from Sigma Chemical Co., St. Louis, Mo., USA. Tumor necrosis factor-α (TNF-α) and fluorescein-conjugated mouse antihuman monoclonal antibodies IgG1 anti-ICAM-1 (CD54), anti-VCAM-1 (CD106) and control IgG1 were purchased from Calbiochem, La Jolla, Calif., USA. Monoclonal antibodies anti-E-selectin (CD62E) and fluorescein goat antimouse IgG $F(ab')_2$ were from Coulter, Immunotech, Marseille, France. An enzyme immunoassay kit for von Willebrand factor (vWF) determination was from RDI, Inc. Flanders, N.J., USA. Equine collagen was from Hormon-Chemie, Germany.

Methods

Platelet Aggregation Studies. Venous blood was obtained from normal individuals using syringes containing ACD anticoagulant (85 m*M* trisodium citrate, 71.4 m*M* citric acid, 111 m*M* dextrose) in a proportion of 1:7 (v/v).

Preparation of Washed Platelets. Suspensions of washed platelets were prepared as described elsewhere [6] with slight modifications. Immediately after collection, blood samples were centrifuged at 190 *g* for 15 min at room temperature to obtain platelet-rich plasma (PRP). PGE_1 (200 µg/ml solution in ethanol) was added to PRP (final concentration 0.25 µg/ml), remaining at 37 °C for 15 min. Then PRP was centrifuged at 1,700 *g* for 15 min, and the platelet pellet was carefully resuspended in modified Tyrode's solution [137 m*M* NaCl, 2.7 m*M* KCl, 0.42 m*M* NaH_2PO_4, 0.35 g% (w/v) bovine albumin, 10 m*M* Hepes, 5.55 m*M* dextrose, 1 m*M* $MgCl_2$, 0.05 µg/ml PGE_1, pH 6.2]. After remaining at 37 °C for 10 min, the suspension was centrifuged at 1,700 *g* for 15 min. The procedure of washing platelets was repeated once again, and finally platelet pellets were resuspended in Tyrode's solution containing calcium [137 m*M* NaCl, 2.7 m*M* KCl, 0.42 m*M* NaH_2PO_4, 0.35 g% (w/v) bovine albumin, 10 m*M* Hepes, 5.55 m*M* dextrose, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, pH 7.4]. Platelet count was adjusted to 300×10^9/l and the suspension was maintained at room temperature until testing.

Aggregation Experiments. Platelet aggregation assays were determined turbidimetrically [7] using a Chronolog aggregometer (model 560). A 400 µl aliquot of the washed platelet suspension was placed in the aggregometer cuvettes, and allowed to warm to 37 °C for 5 min. Then, the agonist solution containing Lopap or buffer was added and the aggregation curve was recorded for 5 min. Final concentrations of the agonists used for platelet aggregation were 50 µ*M* ADP

and 300 μg/ml fibrinogen, 2.5 μg/ml equine collagen, 0.19 U NIH/ml thrombin, 0.1 U/ml human prothrombin, 14.1 μg/ml Lopap.

Studies of Thrombin Generated by Lopap in a Purified System

The amidolytic activity of the thrombin generated by Lopap was determined using the chromogenic substrate S-2238 in a purified system, as well as by its inhibition by AT. The time courses of prothrombin activation were evaluated after preincubation for 10 min at 37°C of 2.5 μ*M* purified Lopap with 90 n*M* prothrombin in the presence or absence of 4.1 μ*M* AT, with 2.5 m*M* $CaCl_2$ to a final volume of 100 μl. The reaction was carried out in 100 m*M* Tris-HCl, pH 8.3. The hydrolysis of 40 μ*M* S-2238 by thrombin formed by Lopap was followed spectrophotometrically in a Spectra MAX 190 (Molecular Devices) spectrophotometer at 405 nm during 20 min at 37°C.

Endothelial Cell Studies

Cell Cultures. Endothelial cells were obtained from HUVEC by collagenase digestion according to the method of Jaffe et al. [8]. HUVEC were identified by their cobblestone morphology and the binding of vWF antibody. Cells were grown in RPMI-1640 medium supplemented with FBS (10%), heparin (90 μg/ml), endothelial supplement growth factor (50 μg/ml), pyruvate (1 m*M*), glutamine (2 m*M*), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator. HUVEC used routinely for experiments were between the first and third passage.

To examine the effect of Lopap, HUVEC were plated on 12-well dishes coated with 2% gelatin and grown to confluence. In order to avoid the interference of heparin or growth factors, complete medium was removed and after three washes, cells were incubated for 1 h in RPMI containing glutamine, antibiotics and different Lopap concentrations. TNF-α was used as a positive control.

Expression of Cell Adhesion Molecules by Flow Cytometry. After treatment of HUVEC with Lopap, medium was removed and after washing, cells were further cultured for 4 h in RPMI containing FBS (10%). Cells were harvested by treatment with 0.25% trypsin-0.02% EDTA solution. After washing with Ca^{2+}/Mg^{2+}-free PBS containing 10% FBS (PBS/FBS), pellets were resuspended in 50 μl of PBS/FBS containing saturating concentrations of FITC-conjugated CD54, CD106, or equivalent concentrations of an isotypic control IgG1. E-selectin was detected by staining cells with CD62E for 1 h at 4°C. The cells were then washed and incubated with goat antimouse IgG FITC-conjugated $F(ab')_2$ fragments (1:100). Cells were fixed with1% paraformaldehyde, and analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, Calif., USA). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls. The numbers of fluorescent molecules per cell were indirectly measured by assessing the mean intensity of arbitrary units of fluorescence (AUF) of cells.

vWF Production. The release of vWF into the culture medium was measured quantitatively by an enzyme immunoassay. Microtiter plates were coated overnight at 4°C with 100 μl purified goat antihuman vWF antibody (3 μg/ml in 50 m*M* Na carbonate, pH 9.6). The remaining protein-binding sites were blocked with 150 μl of blocking solution. After washing, samples and standards diluted in HEPES-Tween-BSA solution were added and incubated for 120 min at 22°C. Then a second anti-vWF antibody conjugated with horseradish peroxidase was added for another 90 min. Binding of the secondary antibody was detected by the addition of 100 μl of [2,2′-azino-di-[3-ethyl-benzthiazcline sulfonate] and the absorbance was read at 405 nm. Results are expressed in ng/ml and were extrapolated from a standard curve constructed from serial dilutions of normal pooled plasma, assuming a vWF concentration of 10 μg/ml.

Statistical Analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using Student's t test for paired data. Differences with a p value <0.05 were considered significant.

Results

Platelet Aggregation

Washed platelet aggregation induced by different agonists was not affected by preincubation of platelets with Lopap. On the other hand, thrombin formation from prothrombin by Lopap was able to induce platelet aggregation similarly to that induced by purified thrombin used as control. These results are shown in figure 1.

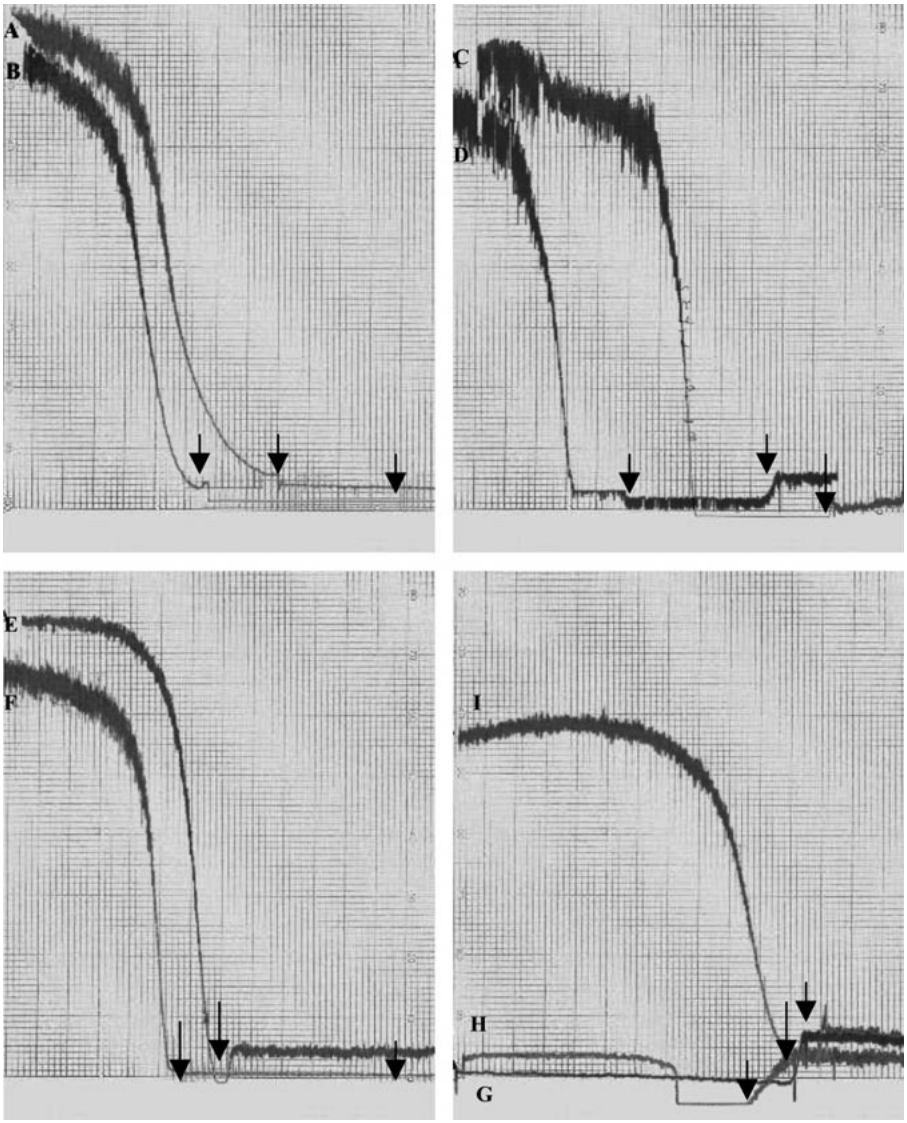

Fig. 1. Washed platelet aggregation after addition of Lopap and different agonists. Arrows indicate agonist addition. A = Thrombin; B = Lopap and thrombin; C = ADP; D = Lopap and ADP; E = collagen; F = Lopap and collagen; G = prothrombin; H = Lopap; I = Lopap and prothrombin.

Inhibition of Thrombin Generated by Lopap

Prothrombin (90 n*M*) incubated with Lopap (2.5 μ*M*) generated thrombin which was able to hydrolyze 40 μ*M* of S2238 chromogenic substrate. Adding AT blocked the amidolytic activity of α-thrombin as well as that of thrombin generated by Lopap (fig. 2).

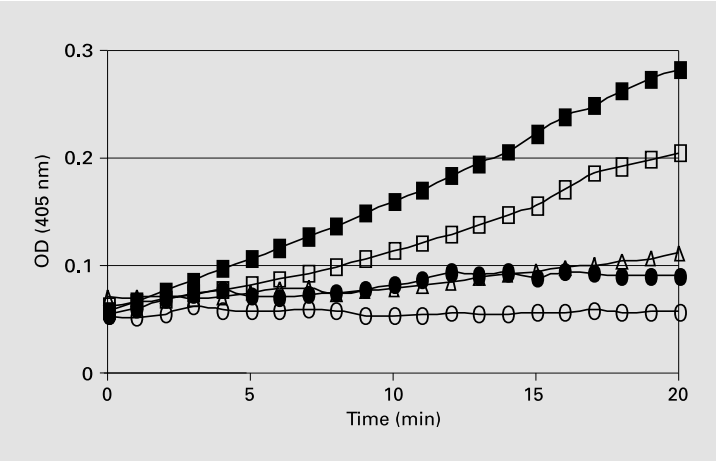

Fig. 2. Amidolytic activity of thrombin generated after Lopap action on prothrombin in a purified system, and its inhibition by AT. ○ = Prothrombin control; □ = prothrombin and Lopap; ■ = thrombin; △ = prothrombin, Lopap and antithrombin; ● = thrombin and AT.

Endothelial Cell Surface Adhesion Molecule Expression

Exposure of HUVEC to Lopap resulted in a concentration-dependent expression of ICAM-1 and E-selectin without detectable increases in VCAM-1 levels, as shown in figure 3. ICAM-1 was still detected 24 h later, whereas E-selectin could not be detected by this time.

Inhibition of Lopap-Induced Cellular Adhesion Molecules

The increased expression of adhesion molecules was specifically mediated by Lopap since preincubation of the purified protein with a serum against whole venom almost completely abrogated E-selectin increase induced by Lopap (fig. 4). Inhibition was not related to an unspecific effect of the serum or the immune complex formed, since the presence of serum did not modify constitutive or E-selectin expression induced by TNF-α (data not shown).

VWF Release Induced by Lopap

While incubation of HUVEC with α-thrombin for 1 h increased the amount of vWF in culture medium, Lopap was not able to modify vWF production (fig. 5). Moreover, levels of vWF after 24 h of endothelial cell treatment with Lopap for 1 h showed results similar (data not shown) to control samples, suggesting that Lopap was not able to induce either vWF release or de novo synthesis of vWF by endothelial cells.

Discussion

A remarkable finding in patients with envenomation by contact with *L. obliqua* is the contrast between a severe consumption coagulopathy, with fibrinogen depletion and production of high levels of prothrombin fragment 1 + 2, thrombin-antithrombin complexes, D dimer, but a modest fall in platelet count and AT concentration [4].

Similar results were observed after infusion of Lopap into rats, showing an almost complete fibrinogen depletion while platelet count was reduced only by 30%. In these animals, collagen-induced platelet aggregation was completely inhibited [3].

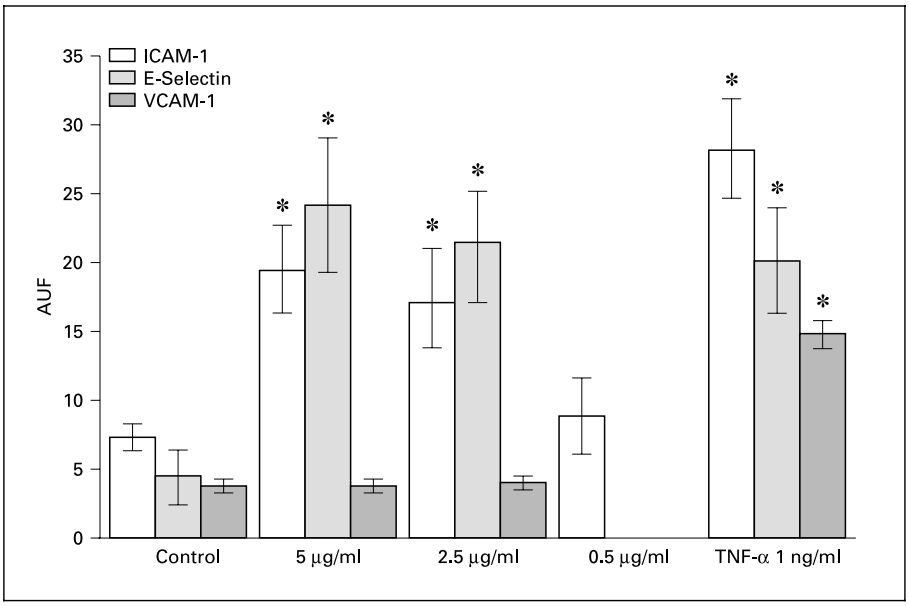

Fig. 3. Expression of ICAM-1, E-selectin and VCAM-1 by HUVECs exposed to different concentrations of Lopap.

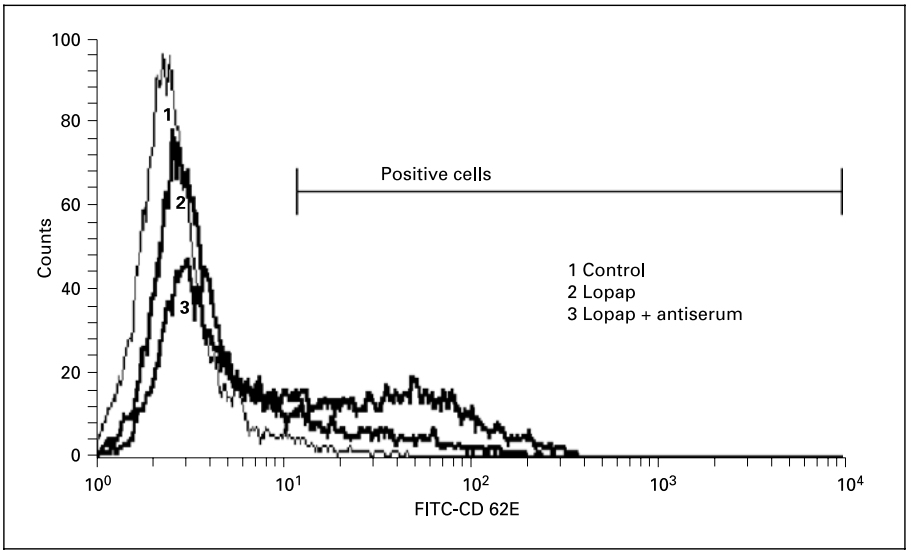

Fig. 4. Inhibition of Lopap-induced HUVEC E-selectin expression by an antiserum against Lonomia.

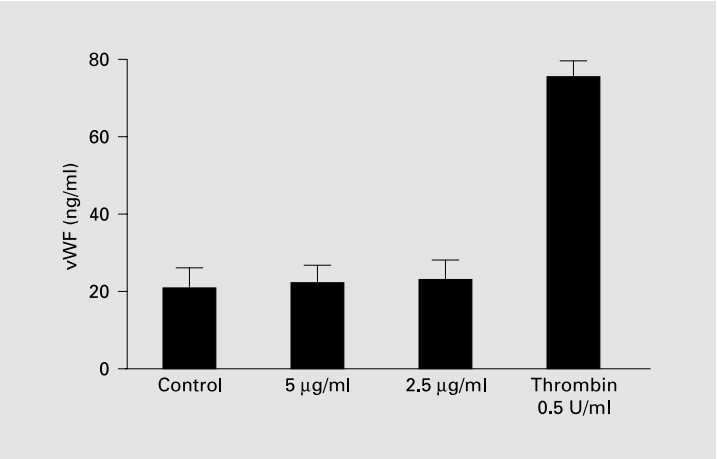

Fig. 5. vW factor levels in the supernatant of HUVEC incubated with different concentrations of Lopap for 1 h at 37 °C.

In the present study, Lopap showed no effect on platelet aggregation induced by collagen, ADP or thrombin, suggesting that it has no direct effect on platelet function.

Indeed, thrombin generated by the action of Lopap on prothrombin was able to induce a normal platelet aggregation response, showing that it can be recognized by platelet receptors.

It is possible that the platelet response to collagen was abolished in rats infused with Lopap because they had been previously activated by thrombin formation and possibly were already degranulated when used in ex vivo experiments.

On the other hand, fibrin degradation products can occupy the GPIIbIIIa fibrinogen receptors and contribute to platelet dysfunction [9].

Circulating activated platelets are not consumed, which can explain the modest fall in platelet count, both in animals and humans, despite a severe consumption coagulopathy.

Thrombin molecules generated by the action of Lopap on prothrombin are able to activate platelets normally and interact with AT as does the α-thrombin formed upon the action of factor Xa on prothrombin.

Why platelet count and AT levels were not reduced in animal models and in human envenomation is not explained. Analysis by mass spectrometry and sequencing of prothrombin fragments induced by Lopap could help to clarify the structural properties of the generated thrombin.

In our study, we used a purified system and we observed that AT was able to block the amidolytic action of the thrombin generated from prothrombin by Lopap, suggesting that it is quite similar to thrombin generated by factor Xa. However, this finding may not represent what happens within the prothrombinase complex on the platelet surface. The direct action of Lopap on endothelial cells might have an important role in the pathogenesis of the coagulation and inflammatory disorders observed during infusion of Lopap in rats or in human accidental contact with *L. obliqua*.

In fact, we observed that Lopap upregulates ICAM-1 and E-selectin expression on cultured endothelial cells, whereas it does not modify VCAM-1 expression.

This effect was specific for Lopap, since serum raised against whole venom almost

completely suppressed E-selectin expression mediated by Lopap.

These results confirm that Lopap not only activates the coagulation system but also triggers endothelial cell responses.

It has been demonstrated that, in addition to its role in intravascular coagulation, thrombin serves as a critical mediator of the inflammatory process through its ability to induce activation of NF-κBp65, expression of ICAM-1- and ICAM-1-dependent PMN endothelial adhesion [10, 11]. Therefore, upon exposure to the venom, cell adhesion molecule (CAM) levels might be upregulated by a direct effect of Lopap on the endothelial surface and through intravascular thrombin formation mediated by Lopap.

ICAM-1 and E-selectin are essential adhesion molecules required for neutrophil recruitment and endothelial transmigration. The ability of Lopap to induce CAM expression correlates with our previous demonstration that in vivo Lopap infusion produced PMN sequestration in pulmonary microcirculation vessels [3].

On the other hand, Lopap showed no effect on vWF synthesis or release by cultured endothelial cells.

These data suggest that Lopap interacts only with some receptors on endothelial cells, causing specific effects, like the expression of few molecules such as ICAM-1 and E-selectin, but not of VCAM-1 or production of vWF.

In this regard, Lopap may behave differently from other mediators such as thrombin itself or TNF-α.

It would be interesting to evaluate the direct action of Lopap on the endothelial expression of other molecules, such as thrombomodulin, as well as its effects on endothelial cell fibrinolytic activity.

In summary, our results showed that Lopap does not affect washed platelet function in vitro, so that the impaired platelet function observed in animals infused with Lopap may be explained by the action of thrombin instead of a direct effect of Lopap.

Thrombin generation by Lopap shows the same action as α-thrombin on a chromogenic substrate, and can be inhibited by AT in a purified system. However, we do not rule out that it could behave differently from thrombin generated in vivo, by the prothrombinase complex.

Finally, Lopap exerts a direct effect on endothelial cells increasing the surface expression of adhesion molecules which participate in inflammatory responses, but it was not able to stimulate vWF synthesis nor its release from endothelial cells.

Further studies are necessary to elucidate other possible interactions of Lopap that could explain some important aspects of the envenoming and may clarify the hemorrhagic syndrome.

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