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rACLF, A RECOMBINANT SNAKE VENOM METALLOPROTEASE, ACTIVATES ENDOTHELIAL CELLS *IN VITRO*

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ABSTRACT: Snake venom metalloproteases (SVMPs) comprise a family of snake venom toxins responsible for most of local and systemic effects observed during envenomation by snakes from the Viperidae family. The vascular system and more specifically the endothelium seem to be the preferential targets of these proteins. This work describes the effects of rACLF, a recombinant SVMP from *Agkistrodon contortrix laticinctus* on human umbilical vein endothelial cells (HUVECs) *in vitro*. Our results showed that rACLF activates HUVECs by the release of mediators involved in inflammation and hemostasis such as prostacyclin and interleukin-8. We also demonstrated that rACLF increased the expression of ICAM-I and decay accelerating factor (DAF). Moreover, rACLF protects the HUVECs against apoptosis induced by serum deprivation. These results suggest that the endothelial cell activation induced by SVMPs may have a significant role in the development of the local inflammatory lesion observed in Viperidae envenomation.

KEY WORDS: rACLF, snake venom metalloprotease, endothelial cells, inflammation.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The vascular endothelium plays a critical role in the regulation of vascular tone, homeostasis, and in the immune and inflammatory systems. Endothelium has also a central function in controlling leukocyte adhesion and migration by close interactions between circulating cells and endothelium. Leukocyte rolling and migration across the endothelium barrier is controlled by expression of adhesion molecules and chemokines on the luminal surface of the endothelium (6, 8, 15).

Snake venom metalloproteinases comprise a subfamily of zinc-dependent enzymes of varying molecular mass found in large quantities in Viperidae snake venom. SVMPs are responsible for most of the local and systemic effects observed during envenomation such as hemorrhage, skin lesions, necrosis, inflammation and cytokine-dependent inflammatory cells influx (4, 11, 13, 14). SVMPs are divided into four groups (PI, II, III and IV) and subgroups that differ for the presence of additional domains on the carboxyl side of the metalloproteinase domain (4, 12, 17). SVMPs display several activities such as degradation of blood coagulating factors (7, 30), extracellular matrix (ECM) components (4, 14, 23, 26) and cell receptors such as the cell-surface β1-integrin receptor (18). Besides these activities, SVMPs were reported to induce apoptosis in endothelial cells (2, 32).

ACLF is a 23,000-Da non-hemorrhagic metalloprotease from the venom of the snake *Agkistrodon contortrix laticinctus*, (28) with fibrinolytic and fibrinogenolytic activities (25, 29). It belongs to the PI class of SVMPs, since it possesses only the metalloproteinase domain with no additional C-terminal domains. Recently, we have demonstrated that rACLF degrades laminin, fibronectin, thrombospondin and collagen IV *in vitro* (23) and strongly decreases the viability of HeLa tumor cells but not of human fibroblasts. Also, this enzyme significantly increases the expression of growth-related oncogene (GRO) and monocyte chemoattractant protein 1 (MCP-1) chemokines by fibroblasts (23).

In order to achieve a better understanding on the role of metalloprotease activity in envenomation, we analyzed the effect of rACLF (recombinant ACLF) on the release and expression of mediators involved in homeostasis and/or inflammation by HUVECs.

MATERIALS AND METHODS

Materials

His-Bind metal chelation resin was from Qiagen (Valencia, CA, USA). RPMI 1640 culture medium, HAM F12 culture medium, fetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin were purchased from Cultilab (Campinas, SP, Brazil). Collagenase was obtained from Worthington Biochemical Corporation (New Jersey, USA) and gelatin was purchased from ICN Biomedicals Inc. (Ohio, USA). Endothelial cell growth supplement (ECGS) from bovine neural tissue, heparin, L-glutamine, 2-mercaptoethanol, sodium pyruvate, vanadium chloride, sodium nitrate, polymyxin B, isopropyl thiol-β-D-galactopyranoside (IPTG), imidazole and urea were from Sigma Chemical Co. (St Louis, MO, USA). Tumor necrosis factor-alpha (TNF-α) and PE-conjugated mouse anti-human monoclonal antibodies (MoAbs; IgG1) against ICAM-1 (CD 54), DAF (CD 55) or control IgG1 were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Enzyme immunoassay kits (ELISA) for prostacyclin (PGI_2) determinations were from Cayman Chemical Company (Ann Arbor, MI, USA) and interleukin (IL)-8 was from Oncogene Research Products (San Diego, CA, USA).

Expression, Purification and Refolding of rACLF

The open reading frame (ORF) coding for pre-ACLF was isolated from a venom gland cDNA library of *Agkistrodon contortrix laticinctus* and subcloned on the pET28a vector (25, 29). Expression, purification and refolding of the recombinant protein were performed as previously described (25). Briefly, transformed cells of *Escherichia coli* strain BL21 (DE3) were grown at 37ºC in Luria-Bertani medium, supplemented with kanamycin (30μg/ml) to a cell density of A660nm=0.4–0.6. After induction of protein expression with 1mM IPTG for 2h, cells were disrupted by sonication and inclusion bodies were solubilized in 20mM Tris-HCl buffer, pH7.9, with 5mM imidazole plus 500mM NaCl with 6M urea. The lysate was centrifuged (10,000X*g*) and the supernatant was applied onto a Ni-NTA resin equilibrated with the same buffer. After washing with buffer with 20mM imidazole, the recombinant protein was eluted with the buffer containing 1M imidazole.

The purified protein was diluted with reducing solution (10mM DTT in 50mM Tris-HCl, pH8.5, with 6M urea) and then diluted with oxidation buffer (50mM Tris-HCl, pH8.5,

5mM cysteine, 1mM cystine, 5mM $CaCl_2$, 100μM $ZnCl_2$ and 6M urea). After dialysis with decreasing concentrations of urea, the solution was concentrated by high-pressure filtration in Amicon (MWCO 10kDa) and the protein concentration was determined by the Bradford method (5). *In vitro* activation of pro-enzyme was carried out by incubating the zymogen at 37ºC for 1h and analyzing by SDS-PAGE.

Endothelial Cell Culture

HUVECs were obtained by collagenase digestion of umbilical veins according to the method of Jaffe *et al*. (16). Cells were seeded on 2% gelatin-coated 25cm^2 tissue culture flasks and identified by its cobblestone morphology and von Willebrand factor (vWF) staining. Initially, cells were grown in RPMI 1640 medium, supplemented with 10% FBS, heparin (45μg/ml), ECGS (25μg/ml), sodium pyruvate (1mM), L-glutamine (2mM), penicillin (100U/ml), streptomycin (100μg/ml) and 2-mercaptoethanol (50μM), at 37°C, in a humidified 5% CO_2 incubator. Having reached confluence, cells were detached by mild treatment with trypsin-EDTA (0.05% and 0.1mM, respectively) and washed with 10% FBS-supplemented PBS (Ca^{2+}/Mg^{2+}-free; PBS/FBS). HUVECs were used between the first and third passages. Some experiments were performed in the presence of polymyxin B (7μg/ml) to rule out lipopolysaccharide (LPS) interference. In order to exclude interference of cellular debris, supernatants from HUVECs exposed for 1h to rACLF were centrifuged for 10min, 400X*g*, at 4°C.

Cellular Apoptosis Detection

HUVECs were incubated in RPMI containing 10% FBS and were stimulated during 48h with or without rACLF. Adherent and floating cells were pooled, washed and fixed in cold 70% ethanol. After centrifugation, cellular DNA was stained with a propidium iodide solution (100μg/ml) in 0.1% Triton-X100, 1mM EDTA. Nuclear endothelial cell changes and necrosis were evaluated by fluorescence microscopy using acridine orange and ethidium bromide solution (100μg/ml of each one).

IL-8 and PGI_2 Assays

To determine the effect of the protease on the release of Weibel Palade bodies, IL-8 levels were measured by ELISA (Oncogene Research Products) in the supernatants of HUVECs that were treated or not with rACLF for 1h. Then, cells were washed and after addition of fresh medium, the constitutive synthesis of IL-8 was measured 24h

later. IL-8 levels are expressed as pg/ml. Production of 6-keto-protaglandin $F_{1\alpha}$ (PGI_2 stable metabolite) was measured by ELISA (Cayman Chemical Company, MI, USA).

ICAM-1 and DAF Expression

After 1h HUVEC, treatment with rACLF, cells were washed and further cultured at 37°C in RPMI containing FBS (10%) during 12 or 24–48h for ICAM-1 and DAF measurement, respectively. Then, cells were harvested by treatment with trypsin-EDTA solution and, after washing with PBS/FBS, they were centrifuged (10min, 200X*g*, at 4°C), resuspended in PBS/FBS and incubated during 30min at 4°C with saturating concentrations of PE-conjugated anti-CD54, anti-CD55 or equivalent concentrations of an isotypic control. Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytometer (Becton Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 5,000 cells per experiment. The percentage of positive cells was determined by thresholds set using isotypic controls. The number of fluorescent molecules per cell was inferred by assessing the mean intensity of fluorescence expressed as arbitrary units (AUF).

Statistical Analysis

All results are expressed as means ± standard error (S.E.). We used one-way analysis of variance (ANOVA) followed by Dunnett test to analyze data and values of p lower than 0.05 were considered statistically significant.

RESULTS

rACLF Protects HUVECs from Apoptosis

The percentage of apoptotic cells in rACLF-treated cultures was similar to that of control, indicating that this enzyme did not induce HUVEC apoptosis at the dose tested after 48h incubation (Table 1). On the other hand, when cell death was induced by serum reduction (1%), rACLF induced significant protection compared to the control. This protective effect was also shown to be concentration-dependent in cells after 24h of serum deprivation (Figure 1). The best effect (about 60%) was observed with 1,000nM rACLF.

Interleukin-8 (IL-8) Release by HUVECs

IL-8 belongs to CXC chemokines family and is an important chemoattractant for PNM, mainly for neutrophils. It also stimulates neutrophil degranulation and adherence to endothelial cells by CD 11b/CD18. Moreover, IL-8 is able to induce angiogenesis and enhanced endothelial cell survival and proliferation (19). To examine the role of rACLF on endothelial chemotatic properties, the release of IL-8 was analyzed. As demonstrated in Figure 2A, the treatment of HUVECs for 1h with rACLF (200 and 1,000nM) significantly triggered the release of IL-8, similar to the effect of TNF, thrombin or LPS.

Prostacyclin (PGI_2) Release by HUVECs

Since PGI_2 and nitric oxide (NO) are the main platelet inhibitors derived from endothelial cells, the release of PGI_2 by rACLF was evaluated. Figure 2B shows that rACLF significantly increased the release of PGI_2 from HUVECs.

ICAM-1 and DAF Expression

The effect of rACLF on the expression of ICAM-1 of HUVECs was examined by flow citometry. Non-treated cells expressed very low levels of ICAM-1. When the cells were treated with 1,000nM rACLF for 1h, the expression of ICAM-1 was significantly increased (Figure 3A). We also examined the effect of rACLF on the decay-accelerating factor (DAF, CD 55) expression. DAF is a cell-surface protein that prevents the formation and accelerates the decay of C3 and C5 convertases, the central amplification enzymes of the complement cascade (20). DAF can be up-regulated *in vivo* during inflammation or *in vitro* by TNF-α (1). A significant increase in DAF expression was observed following treatment with 1,000nM rACLF (Figure 3B).

Table 1. Percentage of apoptotic cells in rACLF-treated human umbilical vein endothelial cells (HUVECs) estimated by fluorescence microscopy.

	% Apoptotic cells*	
Condition	Control	rACLF (200 nM)
10% FBS	18.6±1.0	17±3.8
1% FBS	51±4.0	41±4.6*

HUVECs were cultured in RPMI containing 10% and 1% fetal bovine serum (FBS) with or without rACLF (200nM) for 48h.

At least 200 cells were scored in each experiment. Values are the mean ± S.E. of six independent experiments.

*p<0.05

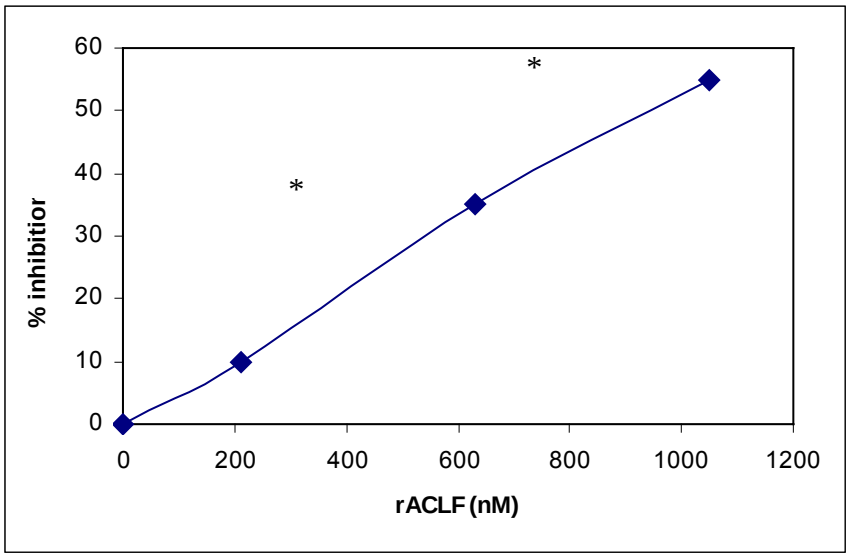
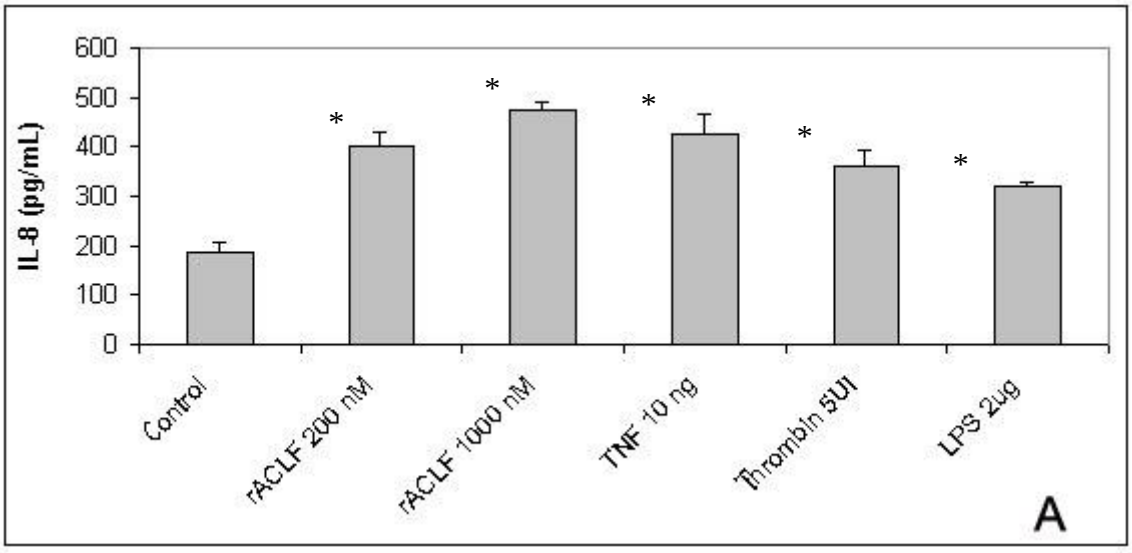


Figure 1. Apoptosis inhibition in human umbilical vein endothelial cells (HUVECs) mediated by rACLF. The HUVECs were treated with rACLF in RPMI medium containing 1% fetal bovine serum for 24h. Apoptotic nuclei were analyzed by fluorescence microscopy. Data represent the means ± S.E. of four independent experiments (p<0.05 versus control).



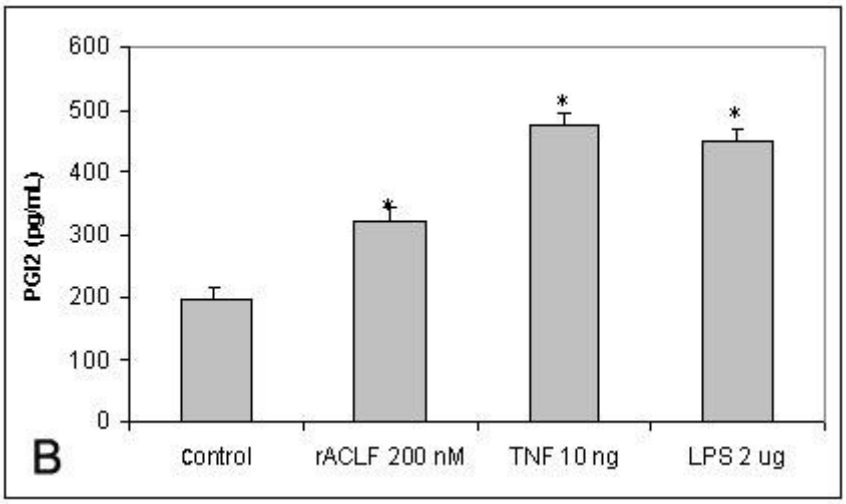


Figure 2. A: IL-8 release by human umbilical vein endothelial cells (HUVEC) treated with rACLF. After 1h incubation of HUVECs with the indicated rACLF, TNF-α or LPS concentrations, IL-8 levels were determined in culture supernatants by ELISA. **B:** PGI_2 production by HUVEC treated with rACLF. After 1h incubation with rACLF, TNF-α or thrombin, 6-keto $PG_{1\alpha}$ levels were determined in culture supernatants by ELISA. Data represent the means ± S.E. of four independent experiments (p<0.01) versus control.

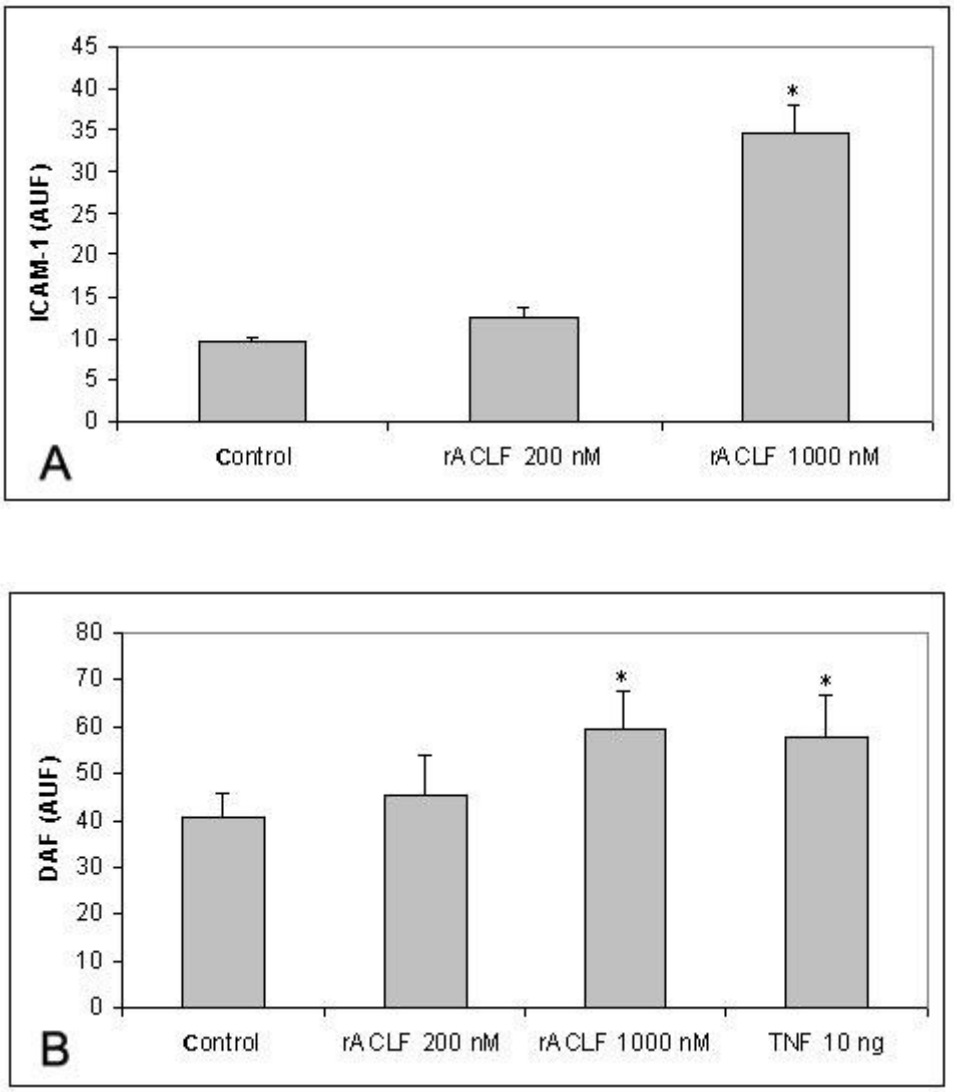


Figure 3. A: ICAM-1 expression by human umbilical vein endothelial cells (HUVEC) treated with rACLF. HUVECs were treated with rACLF for 1h. Then, ICAM-1 expression was determined by flow citometry. **B:** DAF expression by rACLF- or TNF-treated HUVECs for 1h. DAF expression was determined by flow citometry. Data represent the means ± S.E. of four independent experiments (p<0.01) versus control.

DISCUSSION

We have previously demonstrated that rACLF, a recombinant PI SVMP from *A. c. laticinctus*, induces the release of chemokines by human fibroblasts *in vitro* (23). This observation suggests a role for this type of enzyme in the inflammatory reaction observed in snake bites. To contribute to a better understanding of the mechanisms

involved in the pathogenesis of such local lesions, we have studied the effects of rACLF on endothelial cells. Our results showed that rACLF was not cytotoxic to endothelial cells, since it was not able to induce either apoptosis or detachment of HUVECs. We also observed that rACLF protects HUVECs against cell death in a concentration-dependent manner. Control cells cultured in a medium with low FBS died after 24h. However, the addition of rACLF to these cultures significantly enhanced cell survival. Schattner and coworkers also reported similar effect for Berythractivase, a PIII SVMP non-hemorrhagic from *Bothrops erythromelas* that did not modify HUVEC morphology or either induce apoptosis (27). However, Berythractivase had no effect on cell survival. Insularinase A, a PI SVMP from *B. insularis* venom is procoagulant and is not able to induce detachment of endothelial cells. (22)

There are several reports on the ability of SVMPs to induce apoptosis in endothelial cells (9, 21, 32, 34, 35). BaP1, a PI SVMP from *B. asper* with weak hemorrhagic activity induced detachment of endothelial cells by proteolysis of matrix components, resulting in anoikis in endothelial cells (9). Moreover, apoptosis was dependent on BaP1 catalytic activity. Jarharagin, a PIII SVMP from *B. jararaca* also induced anoikis in endothelial cells, but had no effect in fibroblasts or murine peritoneal adherent cells (MPAC) (32).

It has been demonstrated that SVMPs are involved in the pathogenesis of local inflammation by inducing edema and releasing matrix metalloproteases and inflammatory cytokines (14, 24). Our data demonstrate increased levels of IL-8 in the supernatant of rACLF-treated cells. IL-8 is the prototype member of the CXC subfamily of chemokines and can be induced by diverse inflammatory stimuli in many cells (31, 33). Most biological activities attributed to IL-8 are due to its potent chemoattractant activity for neutrophils and its ability to stimulate endothelial cells matrix metallopeptidade expression (19). During the inflammatory reaction, not only occurs an increase in chemokine levels but also an up-regulation of endothelial cell adhesion molecules, which promotes leukocyte recruitment and migration. Leukocyte migration into tissues is a multistep process mediated by three major classes of adhesion molecules: integrins, selectins and immunoglobulins that are expressed by both leucocytes and endothelial cells (3). rACLF up-regulated ICAM-1 expression levels in HUVECs. Similar effect was observed in HUVECs treated with Beythractivase (27, 30).

Fernandes and coworkers reported that BaP1 induced a marked leukocyte infiltration into the mouse peritoneal cavity and this effect was related to the ability of BaP1 to up-regulate the expression of leukocyte adhesion molecules LECAM-1, LFA-1 and CD 18 (11).

Since it was previously demonstrated that SVMPs activate the complement system (10), we further investigated the ability of rACLF to induce the expression of complement inhibitor proteins (CIPs) on the HUVEC surface. Our results showed that rACLF up-regulated DAF expression, which in turn can help in cell protection against complement-mediated cell lysis, reinforcing the response against endothelial injury.

In addition, we demonstrated that rACLF increased the PGI_2 production, a molecule involved in the regulation of vascular tone and potent platelet activation inhibitor. However, more experiments are required to verify the effects of rACLF on platelet function/aggregation.

In conclusion, our results showed that rACLF activates HUVECs by modulating mediators involved in homeostasis and inflammation. Besides that, rACLF is not cytotoxic to endothelial cells and inhibits cell death. Since rACLF belongs to the PI class of SVMPs, our results strongly suggest that the catalytic activity is important for the effects observed in this study. However, the substrate for rACLF and its mechanism of action remains to be elucidated. To our knowledge, this is the first report of a recombinant SVMP activating endothelial cells, therefore modulating inflammation and survival mechanisms.

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