



Lopap: A non-inflammatory and cytoprotective molecule in neutrophils and endothelial cells

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ABSTRACT

Lopap (*Lonomia obliqua* prothrombin activator protease) is a member of the lipocalin family isolated from the extract of *L. obliqua* bristles. Lopap displays serine protease-like activities, including coagulation disturbance, cytokine secretion and antiapoptotic activity in human cultured endothelial cells. Here, we have investigated the effects of the recombinant protein (rLopap) on the inflammatory and apoptotic processes of neutrophils and endothelial cells from male Wistar rats. We found that rLopap did not induce *in vivo* leukocyte–endothelial interactions in the microvasculature, initial steps of leukocyte recruitment during inflammation. Incubation of rLopap with neutrophils or endothelial cells prevented apoptosis evoked by serum deprivation and induced nitric oxide (NO) production in both cell types, and increased the expression of ICAM-1 by endothelial cells. Simultaneous incubation of endothelial cells or neutrophils with rLopap and N^ω-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of NO synthases, inhibited NO production and impaired the protection on apoptosis. Differently, incubation of endothelial cells with monoclonal antibody anti ICAM-1 did not change the protection on apoptosis evoked by rLopap. Together, these results indicate that rLopap does not display inflammatory properties *in vivo* but inhibits apoptosis of neutrophils and endothelial cells depending, at least in part, on NO production.

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1. Introduction

Lopap (*Lonomia obliqua* prothrombin activator protease) is a 69 kDa lipocalin family member with serine-protease like activity, isolated from the crude extract of *L. obliqua* bristles (Reis et al., 1999; Ricci-Silva et al., 2008). *In vivo* and *in vitro* assays have demonstrated that this toxin is an important component of the extract, since both crude extract and purified protein were shown to have similar effects on the blood coagulation cascade. Thrombus formation in microcirculatory vessels of the cremaster

muscle, with consequent fibrinogen depletion, was observed to be dose-dependent after intravenous injection of purified Lopap or crude extract in mice (Reis et al., 1999, 2001). In agreement with this finding, Lopap generates *in vitro* thrombin and pre-thrombin-2, by hydrolyzing the Arg²⁸⁴–Thr²⁸⁵ peptide bond of the prothrombin molecule (Reis et al., 2001).

In addition, it has been shown that crude extract obtained from the bristles induced an inflammatory response in mice, which was characterised by enhanced influx of leukocytes into the subcutaneous cavity (Ramos et al., 2004). Recently, it was demonstrated that the crude extract also induced gene expression of proinflammatory cytokines, interleukin-8 (IL-8), interleukin-6 (IL-6), chemokine ligand-2 (CCL2) and chemokine (C-X-C motif

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ligand 1 (CXCL1) in cultured human fibroblasts (Pinto et al., 2008).

It seems that Lopap may contribute to the inflammatory properties of the crude extract, since incubation of human vein endothelial cells (HUVECs) with the purified protein caused high expression of intracellular adhesion molecule-1 (ICAM-1) and E-selectin (Chudzinski-Tavassi et al., 2001; Fritzen et al., 2005). These molecules are expressed in endothelial cells and are responsible for leukocyte adherence to the vessel wall during diapedesis and intracellular signalling during inflammation (Cook-Mills and Deem, 2005; Hordijk, 2006).

On the other hand, Lopap also evoked the production of nitric oxide (NO) and secretion of prostacyclin-1 (PGI₂) by HUVECs (Fritzen et al., 2005). NO and PGI₂ produced by endothelial cells have been implicated as modulators of the leukocyte migration into inflammatory sites, by acting on leukocyte–endothelial interaction steps (Kubes et al., 1991; Lindemann et al., 2003) and on expression and activity of adhesion molecules expression (Mitchell et al., 1998; Lindemann et al., 2000; Dal Secco et al., 2006). Nevertheless, the role of NO as inhibitor or inductor of the process is controversial and seems to be dependent on source and amount of NO at the site of action (Dal Secco et al., 2003; Hebeda et al., 2008).

Additionally, Lopap prevented HUVEC apoptosis induced by serum deprivation (Fritzen et al., 2005). We have hypothesized that expression of endothelial adhesion molecules and secretion of chemical mediators may participate of the cytoprotective effects of Lopap, as NO modulates apoptosis in several types of cells (Li and Wogan, 2005; Dal Secco et al., 2003; Zeini et al., 2005) and ICAM-1 expression has been correlated to pro- or antiapoptotic effects in different cells, depending on the conditions of disease (Cheng et al., 2008; Martinesi et al., 2008).

Therefore, this work was carried out to evaluate the inflammatory and apoptotic properties of the recombinant Lopap (rLopap) in endothelial cells and neutrophils. *In vivo* and *in vitro* assays were designed using rLopap and male Wistar rats as an experimental model. Data presented herein show that rLopap does not induce leukocyte interaction into endothelium *in vivo* and pro-inflammatory cytokine secretion by neutrophils and endothelial cells, but prevented apoptosis of both neutrophils and endothelial cells, mediated, at least in part, by NO production.

2. Materials and methods

2.1. Reagents

Monoclonal antibodies were purchased from BD Pharmingen Technical (San Diego, CA, USA). *N*-formyl-methionyl-leucyl-phenylalanine, EDTA, oyster glycogen, N^ω-nitro-L-arginine methyl ester, norepinephrine and acetylcholine were purchased from Sigma (St. Louis, Mo, USA). Elisa Kits for IL-6 and TNF- α were purchased from BD Biosciences (San Jose, CA, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, pancreatin and gentamycin reagent solution were purchased from GIBCO BRL Products (Grand Island, NY, USA). Sodium pentobarbital was

purchased from Cristália (São Paulo, Brazil). rLopap was obtained according Reis et al. (2006).

2.2. Animals

Male Wistar rats weighing 180–220 g were used. The animals were fed a standard pellet diet and water *ad libitum*. Before each experimental procedure, the animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) to avoid stress. All procedures carried out on the animals were done according to protocols approved by the local Committee for Ethical Surveillance in Animal Experimentation (COBEA) for the proper use and care of experimental animals.

2.3. Intravital microscopic assay: leukocyte–endothelial interactions and microvascular reactivity

The mesentery of rats was surgically exteriorized and kept on a transparent platform thermostatically controlled at 37 °C to be transilluminated. The tissue was irrigated with a warmed Ringer–Locke solution (pH 7.2–7.4; 154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂·2H₂O, 6 mM NaHCO₃ and 5 mM glucose) containing 1% gelatine. Transilluminated images were obtained by optical microscopy (Axioplan II, Carl-Zeiss, Germany, equipped with $\times 5.0/0.30$ plan-neofluar or $\times 10.0/0.25$ Achroplan longitudinal distance objectives/numeric aperture and $\times 1.0$, 1.25 or 1.60 optovar) and were captured by a video camera (ZVS, 3C75DE, Carl-Zeiss, Germany) connected to the microscope. The images were transmitted simultaneously to a TV monitor and to a computer. Digitized images on the computer monitor were analyzed by image-analysis software (KS 300, Kontron, Germany).

The interaction between leukocytes and vessel walls was evaluated by determining the number of rolling and adhered leukocytes on the postcapillary venule wall (20–30 μ m diameter, 200 μ m length) at 10-min intervals. Quantification was carried out 10 min after topical application of 10 μ l of rLopap at concentrations of 30 μ g/ml, 300 μ g/ml or 1 mg/ml or the equivalent volume of sterile saline. Positive control for the assay was performed by topical application of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP, 10⁻⁸ M, 10 μ l). Three fields from each animal were evaluated. Leukocytes moving in the periphery of the axial stream, in contact with the endothelium, were considered to be rollers. The number of leukocytes stopped at the vessel wall (adhered cells) was determined for the same vascular segments.

2.4. Flow cytometry: adhesion molecules expression and cell viability

Peripheral leukocytes were isolated from blood collected from the abdominal aorta, using EDTA (2 mg/ml) as an anticoagulant. After erythrocytes lysis (ammonium chloride solution, 0.13 M), leukocytes were recovered after washing with Hanks' salt balanced solution (HBSS) and 1 $\times 10^6$ cells were incubated with rLopap (300 μ g/ml, for 15 or 30 min, 37 °C). Incubations with FMLP 10⁻⁸ M were used as a positive control. After being washed, leukocytes were

further incubated for 20 min at 4 °C in the dark with L-selectin or $\beta 2$ integrin monoclonal antibody conjugated with fluorescein isothiocyanate (FITC; 0.5 $\mu\text{g}/10 \mu\text{l}$).

Primary cultures of microvascular endothelial cells were obtained from the cremaster muscle of the rats using the method described by Lotufo et al. (2006). Pure cultures of endothelial cells were identified by expression of PECAM-1 and von Willebrand factor using confocal microscopy. Briefly, the cremaster muscles were isolated from rats and small pieces were placed into a flask and cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 1% gentamycin. After 48 h, the tissues were discarded and the medium was changed. The cells were subcultured with 10% pancreatin in phosphate-buffered saline solution and used for assays on the eighth day, when they achieved confluence. Cells were then incubated with medium or rLopap (300 $\mu\text{g}/\text{ml}$, for 4 or 8 h, 37 °C). Afterward, they were suspended by using a cell scraper and incubated with monoclonal antibody, anti-ICAM-1, anti-VCAM-1 or anti PECAM-1 conjugated with FITC, for 20 min at 4 °C in the dark.

Neutrophils and primary cultured endothelial cells were used to evaluate the effects of rLopap on apoptosis and necrosis. Neutrophils were obtained from the peritoneal cavity 4 h after local injection of oyster glycogen (20 ml of 1% solution) and primary cultured endothelial cells were obtained as described above. After washing with HBSS, 1×10^6 neutrophils or endothelial cells were incubated with DMEM (endothelial cells) or RPMI (neutrophils) containing 1, 10 or 20% of fetal bovine serum (FBS) for 24 h. In order to evaluate the effects of NO or ICAM-1 on the apoptotic activity of rLopap, neutrophils or endothelial cells were simultaneously incubated with N^{ω} -nitro-L-arginine methyl ester (L-NAME, 1 M) and rLopap (300 $\mu\text{g}/\text{ml}$). Endothelial cells were incubated with biotin labeled monoclonal antibody, anti-ICAM-1 (0.5 $\mu\text{g}/10 \mu\text{l}$) and rLopap (300 $\mu\text{g}/\text{ml}$) for 24 h. At the end of treatments, cells were incubated with FITC annexin V (1:500) for 20 min at room temperature in the dark to quantify apoptotic cells. At the moment of flow cytometry analysis, propidium iodide (10 μl of 50 mg/ml solution) was added in order to quantify necrosis.

The quantification of adhesion molecule expressions, apoptosis or necrosis was carried out in a FACScalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data from 10,000 cells were obtained and only the morphologically

viable leukocytes and endothelial cells were considered in the analysis of adhesion molecule expressions.

2.5. Immunoenzymatic assay

Neutrophils or confluent cultured endothelial cells (1×10^6) were incubated with rLopap (300 $\mu\text{g}/\text{ml}$, 500 μl , 18 h) or lipopolysaccharide of *Salmonella abortus* (LPS, 1 mg/ml, 500 μl , 18 h). Concentrations of IL-6 and TNF- α were quantified in the supernatants by immunoenzymatic assays using commercial kits.

2.6. Griess reaction

Neutrophils were incubated with rLopap (300 $\mu\text{g}/\text{ml}$; 500 μl , 18 h) or LPS (1 mg/ml, 500 μl , 18 h) and cultured endothelial cells were submitted to equivalent treatments and incubated for 48 h. The amount of the stable metabolite NO_2^- in the culture supernatants was measured by Griess reaction. Briefly, 100 μl of supernatant was incubated with 100 μl of Griess reagent (sulphanilamide solution 1% and α -naphthyl ethyl diamine solution 0.1%) for 10 min. The absorbance was monitored at 550 nm.

2.7. Data and statistical analyses

Means and standard error mean (s.e.m.) of all data are presented, which were compared by Student's *t*-test or ANOVA, followed by Tukey's Multiple Comparisons. GraphPad Prism 4.0 software (San Diego, CA, USA) was employed. The differences were considered to be significant when *P* was less than 0.05.

3. Results

3.1. Effects of rLopap on in vivo leukocyte-endothelial interactions

Topical application of rLopap to the microcirculatory network of the mesentery did not induce rolling or adherence of leukocytes to endothelium of postcapillary venules (Fig. 1). The concentrations of rLopap employed in this study (30 or 300 $\mu\text{g}/\text{ml}$; 10 μl) did not modify the blood flow or diameter of the vessels (data not shown). Topical

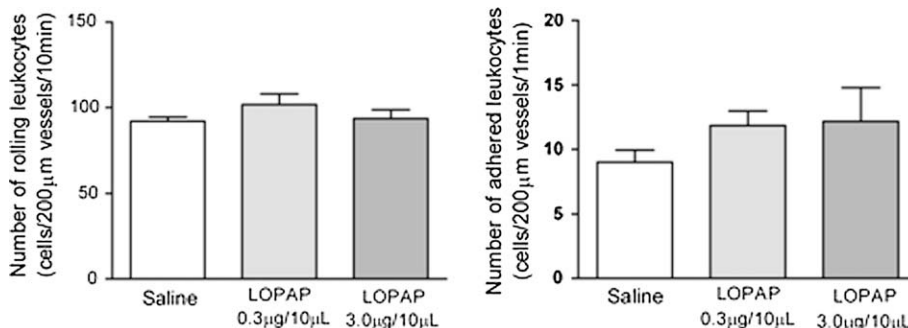


Fig. 1. Effects of rLopap on leukocyte-endothelial interactions in mesentery microcirculation. Rolling and adhered leukocytes in postcapillary venules were quantified 10 min after topical application of sterile saline or rLopap. Data are expressed as mean \pm s.e.m. of six animals in each group.

application of higher concentrations of Lopap (1 mg/ml; 10 μ l) caused thrombus formation at the vessel wall and intermittent stasis of blood flow in the microcirculatory vessels. Similar observations were made after systemic injection of crude extract or purified Lopap in mice (Reis et al., 1999, 2001).

Topical application of FMLP (10^{-8} M; 10 μ l) used in control assays enhanced both events (basal rolling = 120 ± 14 ; FMLP induced rolling = $179 \pm 9^*$; basal adherence: 5 ± 1 ; FMLP induced adherence = $22 \pm 3^*$; $n = 5$; $^*P < 0.05$ vs. basal values).

3.2. Effects of rLopap on expression of adhesion molecules

Circulating leukocytes or primary cultured endothelial cells were *in vitro* incubated with rLopap to quantify the expressions of adhesion molecules by flow cytometry. rLopap did not modify the expression of L-selectin and $\beta 2$ integrin in neutrophils and the constitutive expression of endothelial VCAM-1 and PECAM-1 (Fig. 2). However, rLopap enhanced the expression of ICAM-1 in the membrane of endothelial cells (Fig. 2). It is important to emphasize that expression of neutrophils or endothelial cells induced by FMLP or LPS, respectively, were used as positive controls.

3.3. Effects of rLopap on secretion of pro-inflammatory cytokines and production of NO

Circulating neutrophils represent about 10–20% of leukocytes in the peripheral blood of rats, which impairs the collection of high amount of these cells from blood. Neutrophils migrated to rat peritoneal cavity in response to oyster glycogen have been employed to *in vitro* assays (Mello et al., 1992; Farsky et al., 1997; Lagranha et al., 2007).

Therefore, we employed migrated neutrophils and primary cultured endothelial cells to investigate the role of rLopap on secretion of TNF- α or IL-6 or production of NO. Results presented in Fig. 3 show that rLopap did not induce secretion of both cytokines by neutrophils or endothelial cells. On the other hand, NO production was stimulated by rLopap in both types of cells (Fig. 3).

In all experiments, cells were incubated with known stimulatory agents (FMLP or LPS), as positive controls.

3.4. Effects of rLopap on neutrophils or endothelial cells viability

Cell viability was quantified by necrosis and apoptosis of neutrophils and endothelial cells using flow cytometry. Incubation of rLopap with both cell types did not induce necrosis or apoptosis (Fig. 4A and C). Neutrophils or endothelial cells incubated during 24 h in culture medium containing lower amount of FSB (1%; serum deprivation) were apoptotic. However, simultaneous incubation of rLopap prevented the cell apoptosis caused by serum deprivation (Fig. 4A and C).

3.5. Participation of NO on protective effect of rLopap on apoptosis of neutrophils and endothelial cells

To evaluate the participation of NO on the protective effect of rLopap on apoptosis, neutrophils or endothelial cells were simultaneously incubated with L-NAME and rLopap. Results presented in Fig. 4B and D show that L-NAME treatment abolished or reduced the protective effect of rLopap on neutrophils or endothelial cells apoptosis, respectively. The levels of NO $_2$ in neutrophils incubated with L-NAME were reduced and confirmed the effectiveness of treatment, as demonstrated by the following

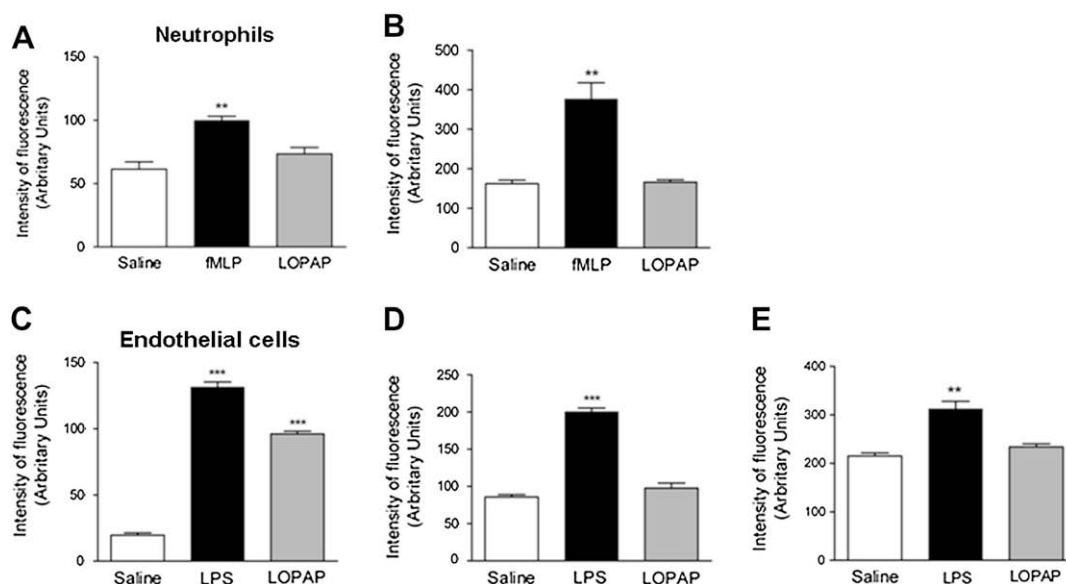


Fig. 2. Effects of rLopap on expression of adhesion molecules in membranes of leukocytes and endothelial cells. Cells were incubated with sterile saline, rLopap (300 μ g/ml) or FMLP (10^{-8} M) and adhesion molecule expression was quantified by flow cytometry. A = L-selectin; B = $\beta 2$ integrin; C = ICAM-1; D = VCAM-1; E = PECAM-1. Data are expressed as mean \pm s.e.m. of cells collected from six animals. *** $P < 0.001$ and ** $P < 0.01$ vs. respective saline-treated cells.

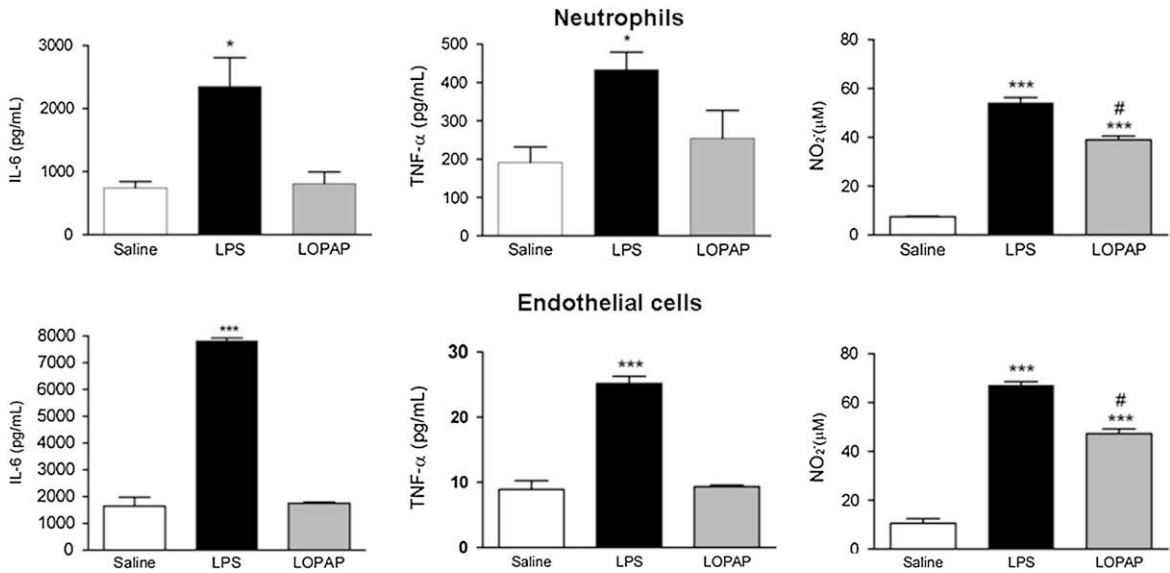


Fig. 3. Effects of rLopap on secretion of chemical mediators by neutrophils and endothelial cells. Cells were incubated with sterile saline, rLopap (300 $\mu\text{g}/\text{ml}$) or LPS (1 $\mu\text{g}/\text{ml}$) and cytokine secretion was quantified by ELISA and NO production by Griess reaction. Data are expressed as mean \pm s.e.m. of cells collected from six animals. * $P < 0.05$ and *** $P < 0.001$ vs. respective saline-treated cells; # $P < 0.01$ vs. LPS-treated cells.

values: serum deprivation = 9 ± 0.30 ; serum deprivation + L-NAME = $6 \pm 0.12^*$; serum deprivation + rLopap = $25 \pm 1.20^{**}$; serum deprivation + L-NAME + rLopap = $10 \pm 0.20^{***}$ ($n = 4$; * $P < 0.05$ and ** $P < 0.01$ vs. serum deprivation; *** $P < 0.001$ vs. serum deprivation + rLopap).

3.6. Participation of ICAM-1 on protective effect of rLopap on apoptosis of endothelial cells

Primary endothelial cells cultured in serum deprived medium were incubated with rLopap and anti-rat

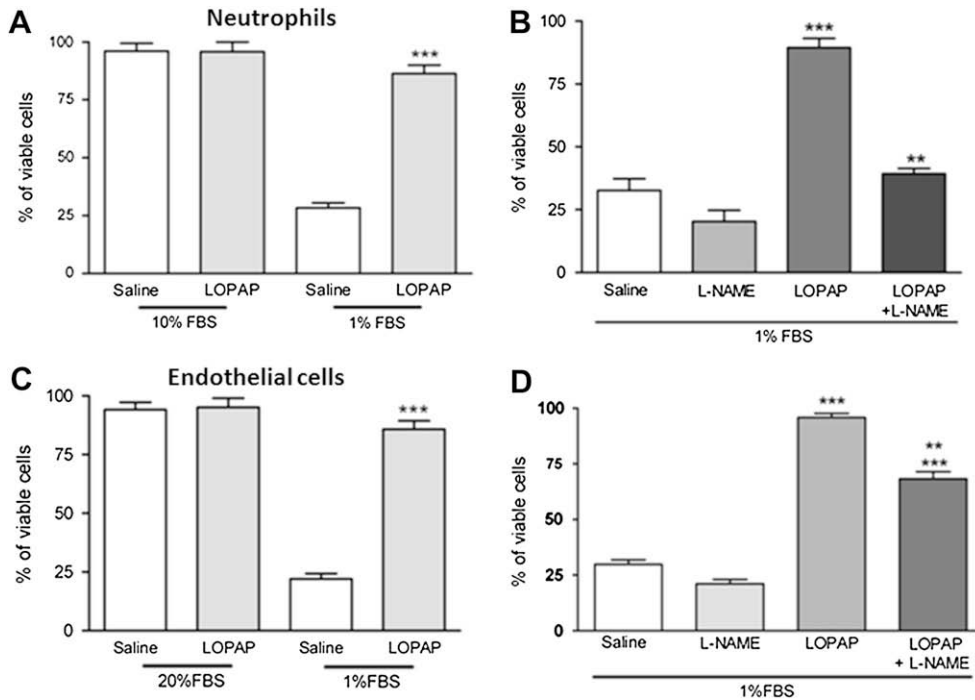


Fig. 4. Effects of rLopap on endothelial cell and neutrophil viability. Cells were incubated with sterile saline or rLopap (300 $\mu\text{g}/\text{ml}$) in: culture medium containing 1, 10 or 20% of FBS (Panels A and C); culture medium containing 1% FBS simultaneously with L-NAME (1 mM, Panels B and D). Apoptotic cells were quantified 24 hours after incubations by flow cytometry using FITC-labeled annexin V and necrotic cells by addition of propidium iodide. Results shown are means \pm s.e.m. of cells obtained from four animals. *** $P < 0.001$ vs. respective saline-treated cells and respective L-NAME-treated cells; ** $P < 0.01$ vs. Lopap-treated cells.

monoclonal antibody against ICAM-1. Percentage of viable cells was similar in cultured endothelial cells treated with rLopap and with rLopap and anti ICAM-1 (Fig. 5).

4. Discussion

Serine proteases are active mediators of diverse biological processes, including digestion of dietary proteins, blood coagulation, and homeostasis. Particularly abundant in animal venoms, serine proteases participate in mobilization and digestion of the prey (Kini, 2005). Based on their biological properties, isolated serine proteases from animal venoms have been used as scientific tools and design models for drug development (Kini, 2005). This work was carried out to elucidate the actions of recombinant serine protease like rLopap, a member of lipocalin family isolated from *L. obliqua* caterpillar, on cellular functions related to inflammation and apoptosis.

Recently it has been demonstrated that serine proteases, especially those involved in the coagulation system such as factor X and thrombin, act via protease-activated receptors (PARs) to signal activation of inflammatory cells. These signalling events mediate motility, cytokine production and adhesion molecule expression by leukocytes and endothelial cells (Roemisch et al., 2002; Pandya et al., 2007; Shpacovitch et al., 2008).

Neutrophils are the first cell recruited into focus of lesion, in a process highly mediated by expression of adhesion molecules in cell membranes, orchestrated by action of chemical mediators secreted by different cells. The initial step of cell recruitment is regulated by three sequential steps initiated by selectin-mediated capturing and rolling, followed by chemokine-triggered activation and integrin-dependent adherence on endothelial immunoglobulin superfamily (Muller, 2003; Alon and Ley, 2008). Data presented here show that rLopap did not induce *in vivo*

rolling or adherence of circulating cells into vessel wall and did not evoke expressions of L-selectin or $\beta 2$ integrin in neutrophil membrane. These results indicate that rLopap does not have a role in the neutrophils interactions into endothelium and suggest that rLopap does not induce inflammatory properties in neutrophils, as enhanced expressions of these molecules are markers of cell activation (Chishti et al., 2004). The inability of rLopap to evoke secretion of pro-inflammatory cytokines TNF- α or IL-6 corroborates this hypothesis. rLopap also did not induce expression of PECAM-1 and VCAM-1 and cytokine secretion by endothelial cells.

Nevertheless, elevated expression of ICAM-1 in endothelial cell membrane and enhanced production of NO was observed in neutrophils and endothelial cells incubated with rLopap. The induction of NO production and ICAM-1 expression by Lopap had already been demonstrated in HUVECs (Fritzen et al., 2005). Here we corroborated these results using the recombinant protein and primary cultured endothelial cell obtained from rat microvasculature and describe, for the first time, the action of the toxin on NO production by neutrophils.

Studies carried out with different cell lineages have demonstrated that NO modulates apoptosis (Moreno et al., 2001; Li and Wogan, 2005; Zeini et al., 2005; Mori, 2007). The action of NO as an inducer of apoptosis is related to its high concentrations and peroxynitrite formation (Guangwu et al., 2008), whereas low levels of NO may function to inhibit apoptosis, by inducing heat shock protein 70 (Hsp70) (Mori, 2007).

We have clearly shown that NO produced as a result of rLopap exposure is associated with the antiapoptotic effects evoked by the protein. Blockade of NO synthases activity by L-NAME treatment inhibited NO production and caused significant blockage and reduction of the anti-apoptotic effect in neutrophils and endothelial cells, respectively.

The role of serine proteases in cell survival and NO production has been examined, mainly by activation of their receptors (Hirano et al., 2007). Tissue factor (TF), factor VIIa and factor Xa promote cell survival by a mechanism involving PAR-2 (Versteeg et al., 2004; Jiang et al., 2006; Versteeg et al., 2008), and thrombin has also been reported as a potent anti-apoptotic factor via PAR-1 activation (Zania et al., 2008). However, induction or inhibition of apoptosis in different cells has been demonstrated after PAR-1 activation, depending on the concentration of the agonists (Flynn and Buret, 2004). Signalling pathways involved in the induction or prevention of apoptosis by PAR-1 activation are diverse, and include JAK/STAT, RhoA, myosin light chain kinase, ERK1/2, and various Bcl-2 family members (Flynn and Buret, 2004). Therefore, the participation of PARs and the intracellular pathways in the rLopap effects here observed will be further investigated.

The mechanism by which thrombin, a serine protease that plays a central role in hemostasis, induces NO production in endothelial cells has been investigated (Thors et al., 2004; Motley et al., 2007). It involves the activation of the constitutive and calcium-dependent nitric oxide synthase (eNOS) through G protein-coupled PAR via Ca^{2+} /calmodulin and protein kinase C δ -sensitive, which

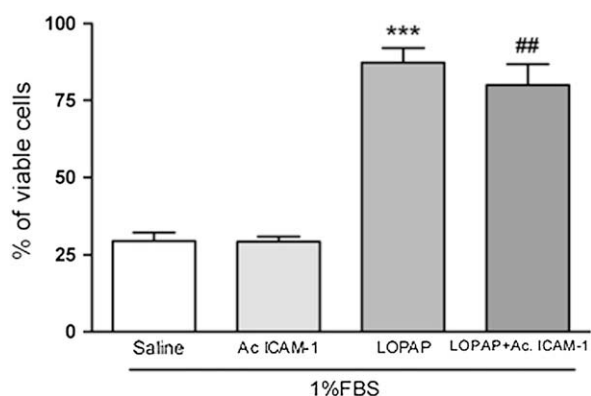


Fig. 5. Role of ICAM-1 expression on rLopap apoptosis protection. Endothelial cells were simultaneously incubated with cultured medium containing 1% FBS and saline; monoclonal antibody anti ICAM-1 (Ac ICAM-1); rLopap (Lopap); and rLopap and monoclonal antibody anti ICAM-1 (Lopap + Ac ICAM-1). Apoptotic cells were quantified 24 hours after incubation by flow cytometry using FITC-labeled annexin V. Results shown are means \pm s.e.m. of cells obtained from four animals. *** P < 0.001 vs. saline; ## P < 0.001 vs. Ac ICAM-1.

cause phosphorylation of eNOS on Ser¹¹⁷⁹ (Motley et al., 2007). In contrast to other agonists, the action of thrombin on eNOS is independent of phosphatidylinositol 3-kinase/Akt-pathway (Motley et al., 2007). The mechanism by which Lopap induces NO production is not understood. Experimental approaches to clarify its action at the transcriptional level or on activation of NOS, as demonstrated by thrombin or other agonists (de Frutos et al., 2001; Fleming and Busse, 2003; Andreeva et al., 2006; Motley et al., 2007), as well as the region of Lopap molecule and the involvement of its catalytic activity on NO production are in progress.

Elevated expression of ICAM-1 in endothelial cell is associated to intracellular signalling in pathophysiological conditions, such as inflammation, lymphocyte trafficking, endothelial contraction and apoptosis (Lehmann et al., 2003; Perez et al., 2007; Vestweber, 2007; Cheng et al., 2008; Martinesi et al., 2008). Here we show that ICAM-1 expressed in endothelial cells by rLopap may not be related to its antiapoptotic effect. The biological significance of expression of ICAM-1 by rLopap remains to be clarified.

Taken together, our findings show that rLopap, a member of lipocalin family with serine protease activities, does not induce *in vivo* and *in vitro* inflammatory properties in neutrophils and endothelial cells, and induces anti-apoptotic effects on endothelial cells and neutrophils, depending on NO production.

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Conflict of interest

There are no conflicts of interest.

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