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Blockade by ruthenium red of tissue factor-initiated coagulation

*,[1]Arthur J. Chu, [1]Zhen-Guo Wang, [1]Obioma I. Nwobi & [2]Salwa Beydoun

[1]Department of Surgery, School of Medicine, Wayne State University, U.S.A. and [2]Detroit Medical Center, University Laboratories, Coagulation Core Lab., University Health Center, 4201 St. Antoine, Detroit, MI 48201 Detroit, MI 48201, U.S.A.

1 The ability of ruthenium red (RuR) to inhibit tissue factor (TF)-initiated blood coagulation was demonstrated at the protein and cellular levels as well as in human plasma.

2 In a single-stage clotting assay, RuR concentration-dependently inhibited rabbit brain thromboplastin (rbTF)-induced coagulation and offset bacterial endotoxin (LPS)-induced monocytic TF (mTF) hypercoagulation; the IC_{50}s were estimated at 7.5 and 12.3 μM, respectively.

3 A 15-min preincubation of RuR with rbTF or monocyte suspension resulted in the pronounced inhibition with a significantly lowered IC_{50} at 1.8 or 7.7 μM for rbTF or mTF procoagulation, respectively. The differences in IC_{50}s between rbTF and mTF without or with the preincubation indicated that TF was a primary target for RuR action.

4 The effect of RuR on the physiological function of TF in FVII activation was demonstrated by the proteolytic cleavage of FVII zymogen to its active forms of serine protease on Western blotting analyses. RuR readily blocked TF-catalyzed FVII activation (diminished FVIIa formation), thus down regulating the initiation of blood coagulation.

5 Inclusion of RuR into human plasma samples *in vitro* significantly prolonged prothrombin time, indicating the depressed coagulation. FVII activity was inhibited by 30–60% depending on the dose; as a result, FX activity also decreased. However, RuR showed no effect on thrombin time. Thus, RuR inhibited FVII activation to block the initiation of coagulation.

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Abbreviations: FVII, factor VII; LPS, bacterial endotoxin; mTF, monocytic TF; PT, prothrombin time; rbTF, rabbit brain thromboplastin; RuR, ruthenium red; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TT, thrombin time

Introduction

Apart from the classical application as a staining-dye in light microscopy, ruthenium red (RuR; ammoniated ruthenium oxychloride, $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6{\cdot}4H_2O$) has been widely used as a pharmacological tool to regulate intracellular Ca^{2+} activities. These included, for instance, the inhibition of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum of muscles (Sutko & Airey, 1996), ryanodine receptor-mediated Ca^{2+} release (Xu *et al*., 1999), plasma membrane Ca^{2+} pump (Missiaen, 1990), mitochondrial Ca^{2+} uniporter (Matlib *et al*., 1998), and Ca^{2+} binding to its binding proteins (Sasaki *et al*., 1992) and calmodulin (Charuk *et al*., 1990). A recent study (Yamada *et al*., 2000) reported that RuR was able to inhibit smooth muscle myosin-light-chain phosphatase, which was proposed to be the mechanism for Ca^{2+}-sensitive contraction in smooth muscle (Yamada *et al*., 1999). In addition, RuR exerted actions on Na+ (Neumcke *et al*., 1987) or K+ (Lin & Lin-Shiau, 1996) channel. A variety of interference with neurotransmission resulting from the wide range of RuR modifications on ion channels was also documented (Trudeau *et al*., 1996; Wang *et al*., 1996; Tapia & Velasco, 1997).

In the present study, we describe a novel biological function of RuR in the blockade of blood coagulation. The enhanced tissue factor (TF)-initiated coagulation often resulting from sepsis or inflammations plays a major role in thrombogenesis, presenting threats of the development of disseminated intravascular coagulation. The upregulated monocytic TF (mTF) activity was primarily responsible for hypercoagulation (Rivers *et al*., 1975; Bertina *et al*., 1989; Chu *et al*., 1999a). RuR antagonized TF-catalyzed FVII activation to inhibit the initiation of blood coagulation, presenting anticoagulant potential.

Methods

Materials

$CaCl_2$, NaCl, Tris-HCl, bacterial endotoxin (*E. Coli* 0111: B04; lipopolysaccharide; LPS), HEPES and horseradish peroxidase-conjugated goat anti-sheep IgG were supplied by Sigma Chemical Co. (St Louis, MO, U.S.A.). Human factor VII (FVII) and FVIIa were from Enzyme Research Lab. Inc. (South Bend, IN, U.S.A.). Sheep anti-human FVII (anti-hFVII Ab) was from Accurate Chemical & Scientific Co. (Westbury, NY, U.S.A.). Pooled normal human plasma and FVII-deficient plasma were from George King Biomedical

*Author for correspondence at: 416 Lande Medical Research Building, 550 E. Canfield, Detroit, MI 48201, U.S.A.
E-mail: ad5742@wayne.edu

Inc. (Overland, KS, U.S.A.). Rabbit brain thromboplastin (rbTF) was from Dade Diagnostics International (Miami, FL, U.S.A.).

Cell cultures and treatments with LPS

Human leukaemic promonocytes (THP-1 cells) purchased from American Type Culture Collection were maintained in RPMI 1640 medium containing penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), 10 mM HEPES and 5% foetal bovine serum (FBS) in a 95–5% air–CO_2 humidified atmosphere at 37°C. FBS was inactivated by heat at 56°C for 30 min. These non-adherent cells were passaged with an equal volume of fresh medium weekly. Prior to experiments, cells were washed with 15 ml serum-free medium followed by centrifugation at $600 \times g$ for 10 min at 4°C. The cells were replated in the fresh complete RPMI and challenged with LPS (100 ng ml^{-1}). At the end of incubation, the cells were washed and resuspended in a TF assaying Buffer **A** containing 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl.

Single-stage clotting assay (Chu et al.*, 2000a)*

TF-initiated coagulation was characterized, and its procoagulant activity was determined by a single-stage clotting assay on a BBL FibroSystem (Becton Dickinson; Cockeyville, MD, U.S.A.). In a fibrin cup containing 50 μl 25 mM $CaCl_2$ (prewarmed to 37°C) and 50 μl either rbTF or cell suspension, RuR prepared in 10 mM PBS (pH 7.4) was included. The reaction was immediately initiated by 50 μl normal human plasma (prewarmed to 37°C). The clotting time in seconds was recorded when the current between two vibrating electric probes was cut off by clots to stop the timer. The stock rbTF (6.5 mg ml^{-1}) arbitrarily assigned as 100,000 units ml^{-1} was assayed to construct a calibration curve ($\log Y = -0.324 \log X + 2.72$; $r^2 = 0.981$) with clotting seconds (Y-axis) and rbTF units (X-axis) being plotted on a log-log graph paper. mTF activity was expressed as units per number of cells determined by the Trypan Blue exclusion test. In some cases, RuR was preincubated with rbTF or cell suspension, reaching the indicated final concentration in the clotting assay.

FVIIa formation on Western blotting (Chu et al.*, 2000a)*

In 100 μl Buffer **A** containing 5 mM $CaCl_2$, FVII (1.5 μg) was incubated with either rbTF (2.1 μg) or THP-1 monocytes (0.85×10^6 cells) at 37°C for 30 min with or without RuR. An aliquot of the sample was diluted in SDS sample buffer and loaded onto a mini-gel subjected to 12.5% SDS–PAGE according to the procedure of Schmidt *et al.* (1984). The protein was transferred onto a nitrocellulose membrane which was blocked by 2.5% dry milk in TBS. The membrane was further immunostained with a sheep anti-hFVII Ab (1 : 250 dilution) for 2 h and a horseradish peroxidase-conjugated goat anti-sheep IgG followed by ECL enhancer (Amersham Life Science) and exposure to X-ray film.

Blood sampling and treatment with RuR in vitro *(Chu* et al.*, 1999a)*

Approximately 5 ml of blood from five healthy non-fasting donors was drawn into an evacuated blood collection tube containing 3.25% sodium citrate. The samples were centrifuged at $2500 \times g$ in a swing bucket rotor for 15 min. Thereafter, the supernate containing plasma was used for performing a variety of clotting assays with or without inclusion of RuR. The individual clotting factors were assayed following the standard clinical laboratory procedures by the in-house clinical laboratory certified by the American College of Pathology. Prothrombin time (PT) was assayed to monitor the initiation of coagulation, while FII was assayed as thrombin time (TT). FVII and FX were assayed, using the corresponding factor-deficient plasma. The per cent activity was also expressed by interpolating the clotting time to the daily corresponding calibration curves ($r^2 \geqslant 0.994 \pm 0.05$).

Data presentation

Statistical analysis was done by SigmaPlot (San Rafael, CA, U.S.A.) with non-paired student's *t*-test.

Results

RuR downregulating TF-initiated coagulation

In cultured THP-1 monocytes, a 3-h exposure to LPS (100 ng ml^{-1}) drastically upregulated mTF resulting in the accelerated mTF procoagulation by nearly 10 fold (Figure 1), which was due to the enhanced mTF synthesis (Chu *et al.*, 2000b). Inclusion of RuR into the single-stage clotting assay offset LPS-induced mTF in a dose-dependent fashion (Figure 1) with an IC_{50} estimated at 12 μM. After a 5-, 10-, or 15-min preincubation of the challenged THP-1 monocytes with RuR,

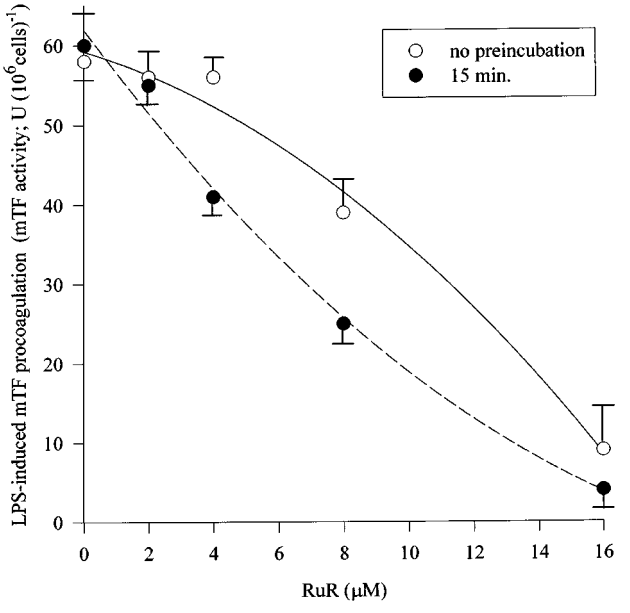

Figure 1 Effect of RuR on mTF hypercoagulation. THP-1 monocytes were challenged with LPS (100 ng ml^{-1}) for 3 h. The cells were harvested and washed. mTF procoagulant activity was assayed as described elsewhere. RuR was included in the assay without or with preincubation with the challenged cells prior to the assay, reaching the indicated final concentration in the reaction mixture. mTF procoagulant activity of resting cells was 6 ± 1 U $(10^6 \text{ cells})^{-1}$ ($n = 4$). The data are means ± s.d. from four independent experiments.

it showed pronounced inhibitions (Figure 1) with the progressively decreased IC_{50} estimated at 9.5, 8.2 or 7.7 μM, respectively (Table 1).

In a cell-free *in vitro* model, RuR effectively blocked rbTF procoagulation (Figure 2). The pronounced inhibition was observed following the preincubation of rbTF with RuR; IC_{50} significantly decreased depending on the duration of preincubation (Figure 2 and Table 1).

Table 2 shows that RuR prolonged PT, indicating the depressed initiation of coagulation. FVII activity decreased as a function of increasing RuR, accordingly resulting in the inhibited FX activity. However, TT remained unaffected.

Ability of RuR to inhibit FVII activation

We examined the effect of RuR on FVII activation. The classical approach to the assessment of FVII activation *via* FVIIa formation for hydrolyzing S-2288 in chromogenic assays was not suitable, because of RuR *per se* showing absorbance at O.D. 405 nm to interfere with the interpretation.

Alternatively, we evaluated FVII activation upon FVII binding to rbTF or THP-1 monocytes by monitoring FVIIa formation immunodetected by anti-hFVII antibody on Western blotting analyses (Figure 3). In THP-1 monocytes, LPS readily enhanced FVIIa formation (lanes 4 *vs* 5) as the result of the elevated mTF synthesis (Chu *et al*., 1999c; 2000b). RuR (10 μM) drastically diminished LPS-induced FVIIa formation (lane 6) to the extent even lower than that in the resting cells (lanes 5 *vs* 6). Similarly, the diminished FVIIa formation was observed upon FVII binding to rbTF in the presence of 5 μM RuR (lane 2). In the corresponding models (e.g., lane 1 *vs* 2 or lanes 3 + 4 *vs* 6), the relative accumulation of FVII zymogen was noted in the presence of RuR.

Discussion

TF-initiated coagulation plays a central role in the revised coagulation cascade, contributing to hemostasis (Broze, 1995). TF is expressed on cell surface upon tissue injuries or inflammation (Ruf & Edgington, 1994; Camerer *et al*., 1996). FVII, FX, and prothrombin activations followed by fibrinogen polymerization constitute this complicated coagulation cascade (Camerer *et al*., 1996; Wildgoose *et al*., 1990; Rao & Rapaport, 1988; Nemerson, 1988; Petersen *et al*., 1995). TF-initiated hypercoagulation could contribute to thrombotic disorder presenting risks for heart attack, stroke or other related coronary heart diseases.

The upregulated mTF activity was shown to be primarily responsible for TF-initiated hypercoagulation induced by bacterial endotoxin (LPS) (Rivers *et al*., 1975; Bertina *et al*., 1989; Chu *et al*., 1999a). It is generally agreed that LPS enhanced TF mRNA content (Gregory *et al*., 1989) resulting in the increased TF synthesis as shown by many laboratories (Bertina *et al*., 1989) including this one (Chu *et al*., 1999c; 2000b). The regulation of LPS-inducible mTF function has been demonstrated at the pretranslational level including the blockade of LPS signalling by n-3 fatty acids (Chu *et al*., 1999b), ethanol (Chu *et al*., 1997), interleukin-4 and 10 (Chu & Prasad, 1998), and the inhibitors of protein kinase C (Chu & Prasad, 1998; Ternisien *et al*., 1993) or tyrosine kinase (Chu & Prasad, 1998; Ternisien *et al*., 1995), all of which could readily lead to the prevention of mTF from upregulation.

Table 1 IC_{50} of RuR on TF procoagulation

	Preincubation (min)			
	None	*5*	*10*	*15*
		(μM)		
rbTF	7.5	3.0	2.1	1.8
LPS-induced mTF	12.3	9.5	8.2	7.7

rbTF and LPS-induced mTF activities were assayed as described elsewhere. Data represent the averages of 4–6 experiments.

Inhibition of mTF-initiated hypercoagulation

We have recently shown the novel anticoagulant potential of compound 48/80 to inhibit TF-initiated coagulation in human peripheral blood monocytes (Chu *et al*., 1999a), cultured THP-1 monocytes (Chu *et al*., 2000b) as well as *in vitro* model involving rbTF (Chu *et al*., 2000a). Apparently, the downregulation of TF function could be of therapeutical value in inhibiting hypercoagulation following inflammation.

Our current results indicated that RuR inhibited mTF-induced coagulation. The similar inhibitory effect was observed in Ca ionophore (A23187)-challenged THP-1 monocytes (data not shown). The inhibitory capability was consistent with the blocked rbTF procoagulation. Importantly, the cell-free *in vitro* model ruled out the possible involvement of RuR cellular regulatory or signalling event(s) in contribution to the anticoagulation.

The data also suggested that RuR was able to inhibit TF function in the initiation of blood coagulation. An indication came from the pronounced inhibition following the brief

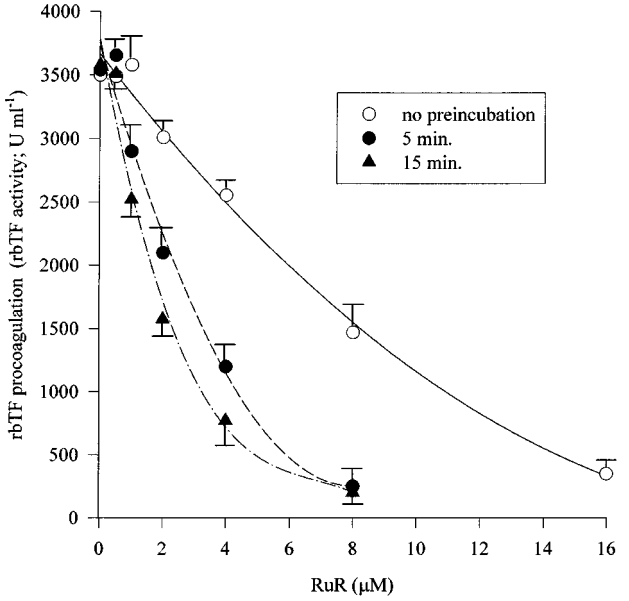

Figure 2 Effect of RuR on rbTF-initiated coagulation. rbTF (2 mg ml^{-1}) procoagulant activity was performed in the single-stage clotting assay as described elsewhere. RuR was included in the assay without or with preincubation with rbTF prior to the assay, reaching the indicated final concentration in the reaction mixture. The data are means ± s.d. from six independent experiments.

Table 2 Effect of RuR on clotting factors in human plasma

Parameter	Saline control	RuR (μM) 2	4	8
PT (seconds)	11.1±0.3	13.2±0.2*	15.1±0.6**	16.3±0.9**
% normal	101±6	72±6	38±6	32±3
FVII (seconds)	19.5±1.2	22.3±0.9*	28.8±1.2**	32.5±2.1**
% normal	92±5	65±3	36±4	31±3
FX (seconds)	18.7±0.2	20.5±0.3	25.2±0.8**	29.6±1.1***
% normal	95±7	71±5	41±6	35±2
TT (seconds)	12.3±0.5	12.4±0.1	13.5±0.8	12.6±0.5

Plasma was freshly prepared as described elsewhere. The samples with the inclusion of the indicated final concentration of RuR were assayed by the in-house clinical laboratory. The data present means±s.e.mean of clotting sceconds as well as per cent normal from five experiments. In the corresponding clotting parameter, *vs* saline ($P<0.05$); **vs saline ($P<0.03$) and ***vs saline ($P<0.02$).

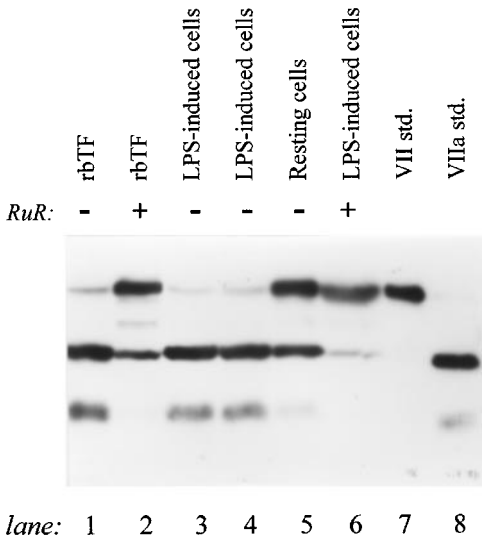

Figure 3 Effect of RuR on FVII proteolytic activation. FVII (1.5 μg) was incubated with rbTF (2.2 μg) in the absence or presence of 5 μM RuR at 37°C for 30 min. In some cases, FVII (1.5 μg) was incubated with the same amount (0.85×10^6 cells) of LPS-challenged cells, resting or LPS-challenged cells in the presence or absence of 10 μM RuR. The samples were subjected to 12.5% SDS–PAGE. The blot was immunostained with sheep-anti-hFVII followed by peroxide-conjugated goat anti-sheep IgG as described elsewhere.

preincubation with RuR. It was true for either rbTF or THP-1 monocytes, revealing the interaction between RuR and TF molecule playing a role. More clearly, the inhibitory action was much efficient on rbTF (Figure 2) than on mTF (Figure 1) buried in the plasma membrane. Under the same single-stage clotting assay, such distinct IC_{50} (Table 1) could only be due to the different accessibility of RuR to TF molecule. Taken together, our data therefore allowed a conclusion to be drawn that TF protein molecule was a primary target for RuR inhibitory action.

Inhibition of FVII activation accounting for the depressed coagulation

The physiological function of TF is known to serve as receptors (CD142 in cytokine receptor superfamily) for FVII/FVIIa (Ruf & Edgington, 1994; Camerer *et al*., 1996). Upon binding FVII activation, a proposed focal event of TF-initiated pathway (Rao & Rapaport, 1988; Nemerson, 1988; Petersen *et al*., 1995), undergoes a proteolytic cleavage between Arg^{152} and Ile^{153} to form its active FVIIa (Wildgoose *et al*., 1990) for the downstream activations of serine proteases in the coagulation cascade (Nemerson, 1988; Petersen *et al*., 1995).

Our previous study showed that the enhanced FVII activation with FVIIa formation consistently dictated the upregulation of mTF-initiated coagulation regardless of THP-1 monocytes being induced by either LPS or Ca ionophore A23187 (Chu *et al*., 2000b). In the current study, it was approximated that rbTF-induced coagulation was 15–20 fold as active as LPS-induced mTF hypercoagulation on the basis of μg protein. The accelerated rbTF procoagulation was apparently attributed to the effective accessibility/binding of FVII to TF molecule, resulting in the enhanced FVII activation. Taken together, evidence pointed to a notion that FVII activation ensured TF procoagulant activity. Our current study is the first to demonstrate the anticoagulant potential of RuR which inhibited TF-catalyzed FVII activation (i.e., diminished FVIIa formation) (Figure 3). The notable accumulation of FVII zymogen further strengthened the view that RuR readily prevented FVII from proteolytic cleavage, thus effectively blocking TF-initiated coagulation.

Increasing evidence (Carson, 1987; Ettelaie *et al*., 1996; Conkling *et al*., 1989; Girard *et al*., 1990; Ruf & Edgington, 1991; Pitlick, 1975) also agreed that the inhibition of FVII activation resulted in the downregulation of coagulation. For instance, 48/80 preferentially depressed FVII activation and FVIIa formation, exhibiting the inhibition of rbTF (Chu *et al*., 2000a) or mTF (Chu *et al*., 2000b) initiated coagulation. Sphingosine in addition to its well-known PKC inhibition has been proposed to insert into membrane to alter FVII binding to mTF (Conkling *et al*., 1989), thereby depressing TF upregulation in LPS-challenged monocytes. Tissue factor pathway inhibitor (TFPI) formed a quaternary complex with TF/FXa/FVIIa, exerting the complicated feedback inhibition (Girard *et al*., 1990). Anti-TF antibody complex with its antigen could prevent the catalytic activity in the initiation of blood coagulation (Ruf & Edgington, 1991). ConA reversibly binding to TF apoprotein possibly induced TF conformational changes unfavourable for FVII binding (Pitlick, 1975). Although apolipoproteins (Carson, 1987; Ettelaie *et al*., 1996), TFPI (Girard *et al*., 1990) or sphingosine (Conkling *et al*., 1989) showed anticoagulation, little is known about whether those 'physiological components' are of therapeutical value.

This study could well serve as a model for the future development concerning specific antagonism to TF-initiated hypercoagulation. FVII activation could be considered a therapeutical targeting of anticoagulation. RuR readily rescued mTF hypercoagulation resulting from a wide variety of inflammation; in fact, the prolonged PT and the depressed FVII/FX activity upon inclusion of RuR into plasma samples *in vitro* (Table 2). Further studies warrant pursuing whether RuR has any clinical application.

Recent efforts were made to study RuR inhibitory mechanism. In viewing of Ca^{2+} being a cofactor throughout the blood coagulation cascade (Broze, 1995; Ruf & Edgington, 1994; Camerer *et al*., 1996; Wildgoose *et al*.,

1990; Rao & Rapaport, 1988; Nemerson, 1988; Petersen *et al*., 1995), the anticoagulation by RuR however was not due to the simple Ca^{2+} chelation/deprivation/caging event. To effectively block TF-initiated clotting, a threshold stoichiometric ratio at 1 : 4, e.g., approximately 1 mM Ca^{2+} chelator (EDTA, EGTA and BAPTA) was required (data not shown). Such a low μM range of RuR could not effectively sequester 4 mM $CaCl_2$, the optimal concentration for TF-initiated coagulation. The molecular mechanism remains unknown, awaiting further study to determine if the inhibition on TF function in FVII activation is associated with any RuR regulatory action on ion-channel functions which, in turn, might be involved in FVIIa formation.

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