

Article

Profiling of Signaling Pathways in Human Dermal Blood and Lymphatic Endothelial Cells Induced by Snake Venom Cysteine-Rich Secretory Protein (svCRiSP) from North American Snakes

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Abstract: Snake venom Cysteine-Rich Secretory Proteins (svCRiSPs) are important components in the venom of many snake species. Little is known about the contribution that they make to the local pathophysiology of snakebite. We investigated the role of svCRiSPs from the most medically significant species of North American snakes (*Crotalus atrox*, *C. adamanteus*, *C. scutulatus scutulatus*, *C. horridus*, and *Agkistrodon piscivorus*), focusing on the cellular and molecular mechanisms. We evaluated the biological activities of svCRiSPs (Catrox-CRiSP, Cada-CRiSP, Css-CRiSP, Chor-CRiSP, and App-CRiSP) by using both in vitro assays on human dermal lymphatic (HDLECs) and blood (HDBECs) endothelial cells permeability and in vivo Miles assay. Of all the CRiSPs tested, Css-CRiSP and App-CRiSP displayed the highest increase in permeability compared to other crotaline CRiSPs. We initially screened the changes in protein expression and phosphorylation in HDLECs and HDBECs after treatment with Css-CRiSP and App-CRiSP using reverse phase protein arrays (RPPA). Studies are ongoing for identifying the key signaling that are involved in endothelial permeability after treatment with App-CRiSP and Css-CRiSP.

Keywords: signaling pathway; reverse phase protein arrays (RPPA); snake venom cysteine-rich secretory proteins (svCRiSPs); endothelial permeability; North American snakes

Key Contribution: Knowledge gained from these studies provides insights into the molecular mechanisms that underlie the effects of svCRiSPs on vascular function and contributes to a new level of understanding of the pathophysiology of snakebite

1. Introduction

Snake venoms are composed of complex mixtures of biological molecules. Among them, snake venom cysteine-rich secretory proteins (svCRiSPs) are ubiquitous components in the venom of many species of snakes [1,2], including Viperidae (Viperinae and Crotalinae) and Elapidae (Elapinae and Hydrophiinae) [3]. However, little is known about the contribution that they make to the local pathophysiology of snakebite. Much of the information that has been garnered on the structure and biological activity of

svCRiSPs has been derived from studies of Asian and Australian snakes (due in part to the concentration of snake venom research in these geographic areas). Much less is known of the biological activity of svCRiSPs present in the North American snakes' venom, especially rattlesnakes (Genus *Crotalus*) and cottonmouths (Genus *Agkistrodon*). Several of these svCRiSPs have been shown to block the activity of L-type Ca^{2+} and/or K^+ channels [4,5]. Two svCRiSPs, ES-CRiSP, isolated from an Asian pit viper, and natrin, isolated from cobra venom, have been shown to affect vascular endothelial cell activity [6,7]. Natrin from *Naja atra* has been shown to produce acute activation of MAP kinase signaling in human umbilical vein endothelial cells (HUVEC) [6]. ES-CRiSP from *Echis carinatus sochureki*, on the other hand, has not been reported to have an effect on MAP kinase signaling on its own but can block VEGF-dependent activation of Erk1/2 in HUVEC cells [7]. However, the mechanism of actions and molecular targets of most svCRiSPs remain unclear.

We have recently reported that Hellerin, a svCRiSP that we have isolated from the venom of the Southern Pacific Rattlesnake, *C. oreganus helleri*, increases vascular permeability in vivo and in vitro [8]. This study's objective was to investigate the biological activities of svCRiSPs isolated from the venoms of the most medically significant species of North American snakes. This study also aims to determine key signaling pathways induced by svCRiSPs on human endothelial cells using reverse-phase protein microarray analysis (RPPA) analysis.

2. Materials and Methods

2.1. Snake and Venom Collection

The venoms were obtained from the National Natural Toxins Research Center (NNTRC), Texas A&M University-Kingsville, Kingsville, TX, USA.

2.2. Purification of CRiSPs

svCRiSPs were isolated from crude venoms using two chromatographic steps consist of reverse phase C18 (Higgins Analytical PROTO 300 C18, 250 × 4.6 mm, 5 μm column, Higgins Analytical, Inc., Mountain View, CA) and cation-exchange (Waters Protein-Pak™ SP 5PW, 7.5 × 75 mm column, Waters Corp., Milford, MA) as previously described [8].

2.3. SDS-PAGE Electrophoresis and N-Terminal Sequencing

Purified CRiSPs were applied to NuPAGE® Novex 4–12% (w/v) Bis-Tris SDS-PAGE gels (Invitrogen™, Carlsbad, CA, USA) using an XCell SureLock™ system (Invitrogen™, Carlsbad, CA, USA). For the N-terminal sequencing, purified CRiSPs were transferred from an SDS-PAGE gel onto a PVDF membrane and stained with Coomassie brilliant blue R-250. The sample membrane was processed for N-terminal amino acid sequencing using Edman degradation method on a PPSQ-33B protein sequencer (SHIMADZU, Kyoto, Japan) following the manufacturer's instructions.

2.4. LC-MS/MS Analysis and Proteomics Data Processing

Venom protein identification and sequence analysis of CRiSPs (Css-CRiSP from *C. s. scutulatus*, Catrox-CRiSP from *C. atrox*, Cada-CRiSP from *C. adamanteus*, Chor-CRiSP from *C. horridus*, and App-CRiSP from *A. p. piscivorus*) were performed by LC-MS/MS mass spectrometry following the protocol as described by Suntravat et al. [8]. N-terminal amino acid sequences and sequences generated by MS were compared to the sequences in the GenBank database using BLASTX programs. Multiple alignments of the complete amino acid sequences were performed with a ClustalW program.

2.5. Effect of CRiSPs on Vascular Permeability and Endothelial Cell Function

Assays were performed as described previously [8]. Values are presented as fold increase with respect to negative control.

2.6. Reverse Phase Protein Array Analysis

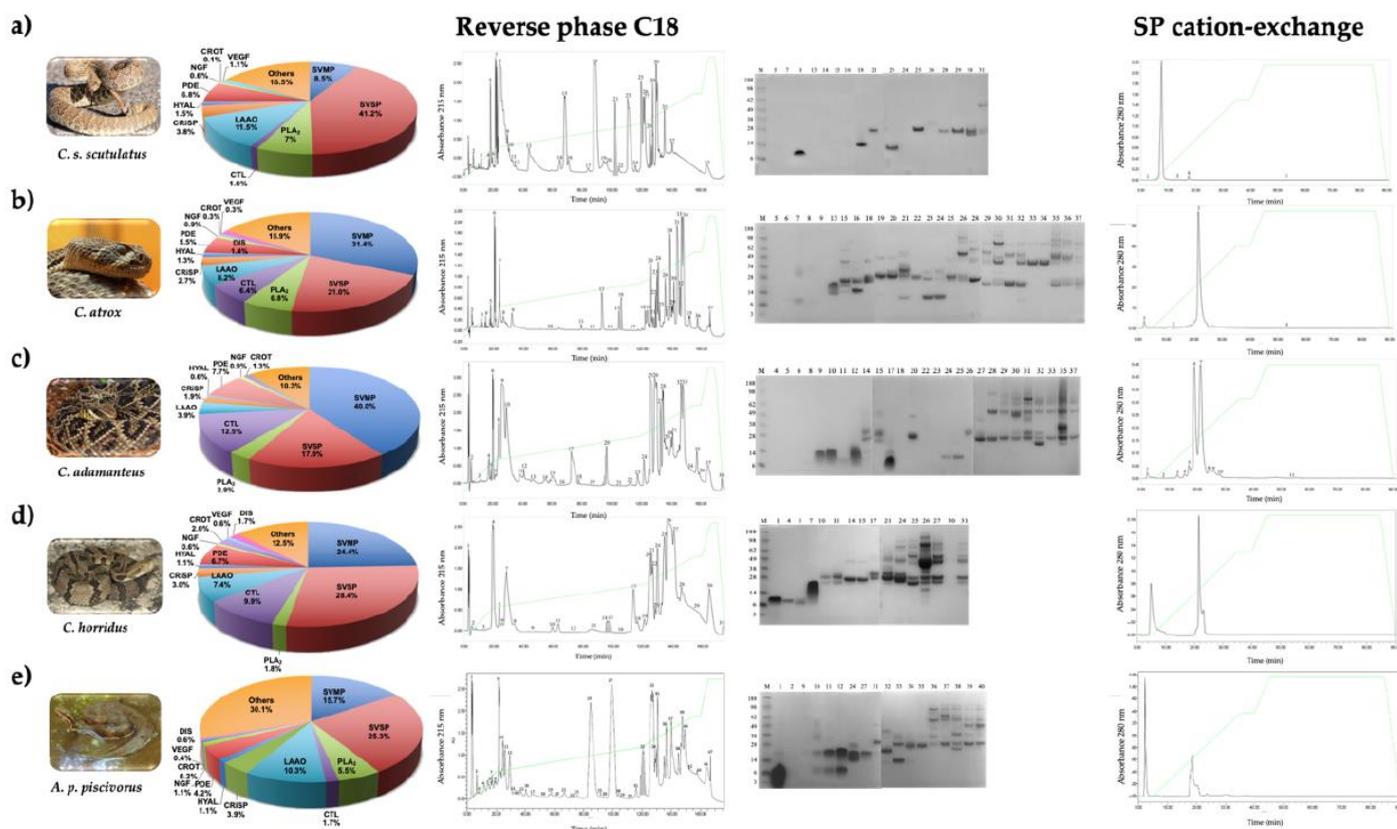
RPPA analyses were performed at the Functional Proteomic RPPA Core Facility at MD Anderson Cancer Center (Houston, USA) as described at <https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/education-and-references.html>. Heatmaps were generated using XLSTAT software version 2020.4.1. Proteins were ordered by the rank sum of the normalized values.

3. Results and Discussion

3.1. Proteomic Analysis and Purification of CRiSPs

Crotaline venoms are a rich and untapped resource for the discovery of new svCRiSPs that are useful in probing the cellular and molecular mechanisms involved in the effects of this family of toxins and on both lymphatic and blood vascular endothelial cell biology. To evaluate the venom composition of the *C. atrox*, *C. adamanteus*, *C. s. scutulatus*, *C. horridus*, and *A. p. piscivorus* used in this study, we analyzed the proteome of the venom using LC-MS/MS analysis. CRiSPs were identified to be 1.9–4% of the total venom proteomes (Figure 1, panels a–e). This observation corresponded to the previous proteomic and transcriptomic studies of these snakes [9–13].

We have successfully purified svCRiSPs from these crotaline venomous snakes using a two-step chromatographic protocol consisting of a reverse phase C18 HPLC chromatography, followed by SP-5PW cation exchange chromatography (Figure 1, a–e). Purified CRiSPs showed molecular mass of approximately 25 kDa under non-reducing conditions (Figure 1f) and were verified as CRiSPs by N-terminal automated Edman Degradation amino acid sequencing.



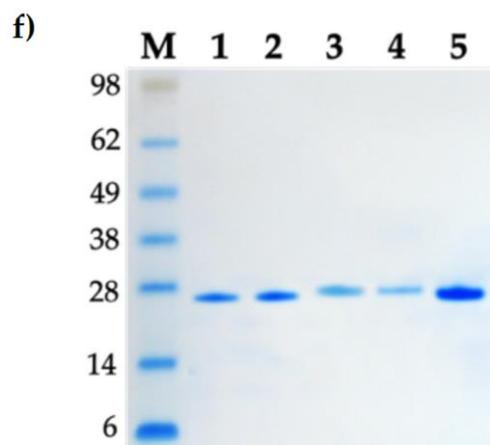


Figure 1. Venomics and purification steps of CRiSPs from five North American snakes (Panels a–e). Relative abundance of different protein families in each venom were identified by LC–MS/MS and are shown in pie charts. (f) SDS-PAGE of purified CRiSPs. M: SeeBlue Plus2 Markers (Invitrogen™). Lane 1: *App*-CRiSP; lane 2: *Catrox*-CRiSP; lane 3: *Csx*-CRiSP; lane 4: *Chor*-CRiSP; lane 5: *Cada*-CRiSP.

3.2. Amino Acid Sequences of CRiSPs

Our CRiSPs shared a high similarity (80%–97%) to each other, and 70%–72% to elapid CRiSPs (Figure 2). Subtle variations in the conformations and amino acid residues could be responsible for their distinct biological functions.

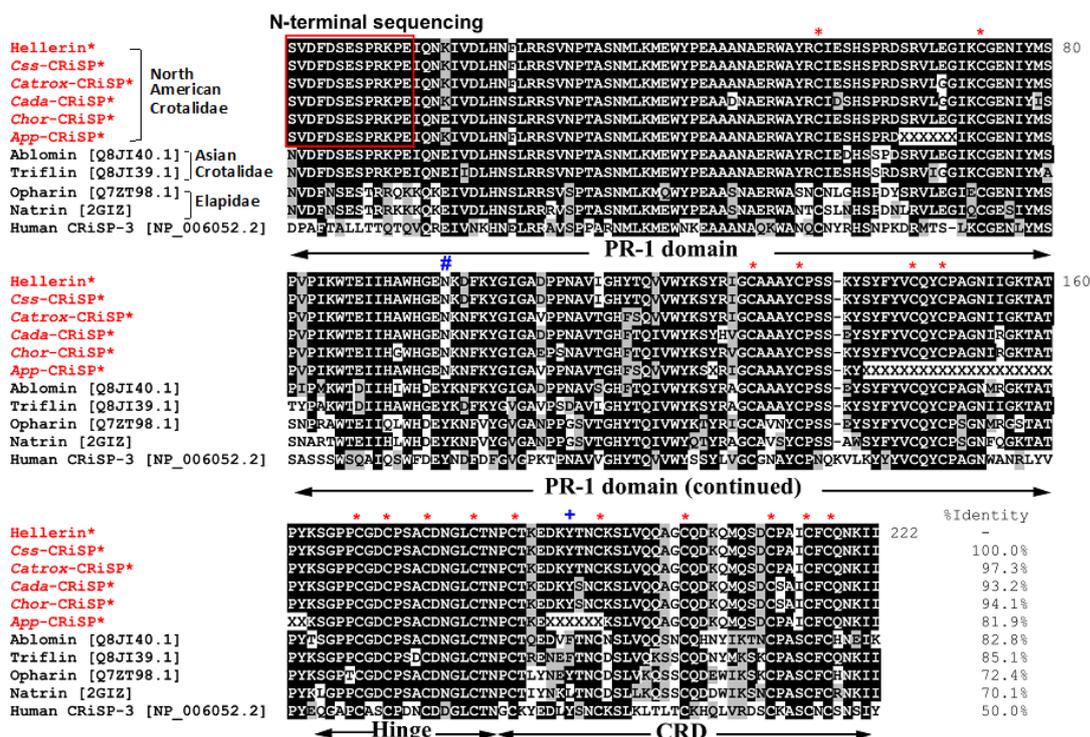


Figure 2. Multiple alignments of the amino acid sequence of svCRiSPs with other homologous venom proteins. All cysteine residues (an asterisk above the sequences) are conserved.

3.3. Effect of CRiSPs on Vascular Permeability and Endothelial Cell Function

In vivo Miles assay, subcutaneous injection of *Cxs*-CRiSP and *App*-CRiSP produced a significant rapid increase (59%) in the trans-capillary leakage of an intravascular dye

(Evans blue) compared to the saline control (Figure 3). App-CRiSP and Chor-CRiSP increased the vascular permeability to 33% and 26%, respectively. Interestingly, neither Catrox-CRiSP nor Cada-CRiSP was active in the Miles assay.

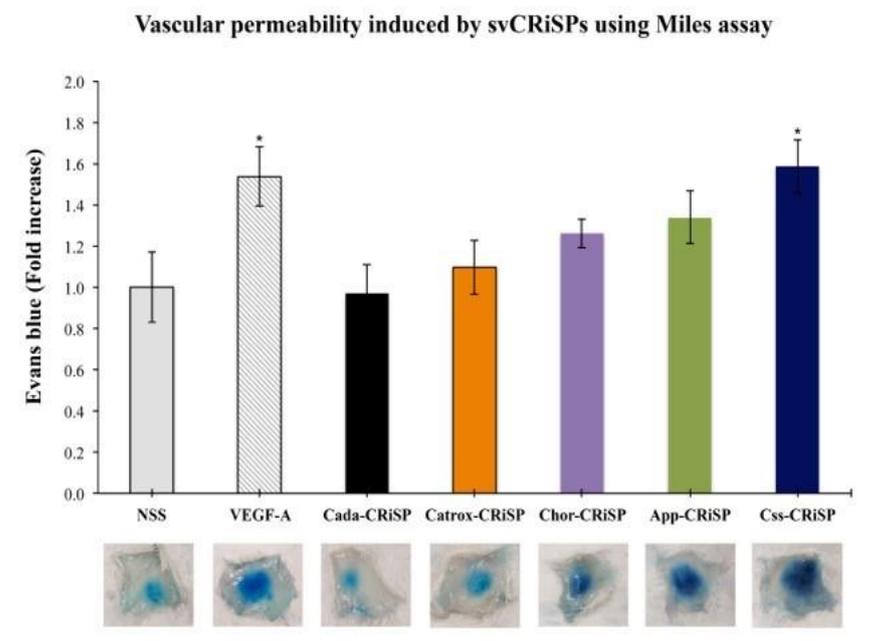


Figure 3. Quantification of an in vivo permeability assay was performed using the modified Miles assay. The results are presented as the fold change in OD for the agent versus vehicle (error bars: SE).

To further analyze the effect of CRiSP on blood and lymphatic cell permeability, we used HDBECs and HDLECs in a cell permeability assay (Figure 4). App-CRiSP, Css-CRiSP, and Catrox-CRiSP caused a significant and dose-dependent increase in HDBEC permeability at 1.3 μM after 1 h of administration (Figure 4a). At 1.3 μM, Css-CRiSP, Catrox-CRiSP, and App-CRiSP but not Cada-CRiSP and Chor-CRiSP, significantly induced trans-endothelial permeability in HDLECs in comparison with that of cells treated with the PBS control (Figure 4b). Of all the CRiSPs tested, Css-CRiSP and App-CRiSP displayed the highest increase in acute vascular and endothelial permeability compared to other crotaline CRiSPs.

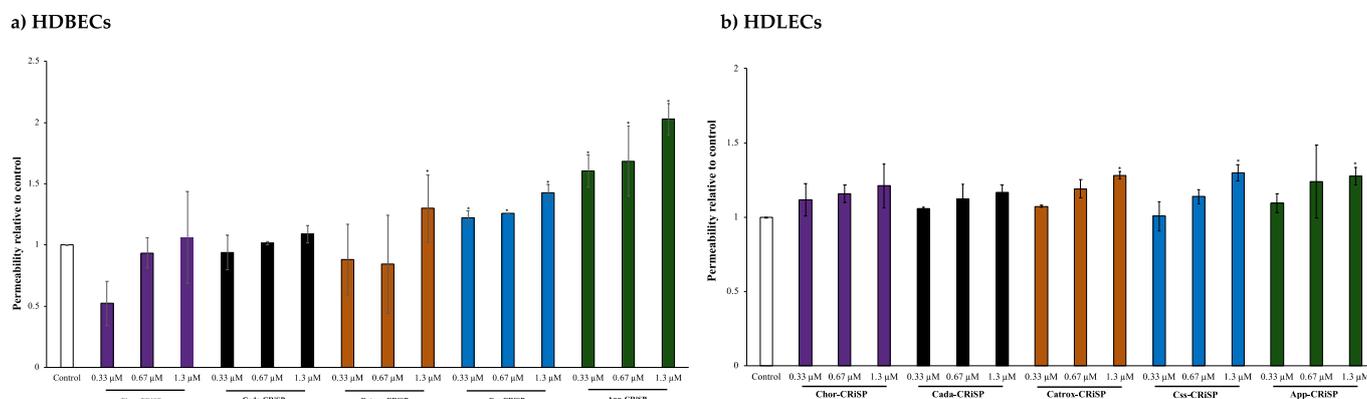


Figure 4. Effect of CRiSPs on monolayer barrier function of HDBECs (a) and HDLECs (b). Data expressed as mean ± SD of two individuals experiments ($n = 3$). * $p < 0.05$, compared with untreated control.

4. Conclusions

This study opens new avenues of exploration on the molecular targets of svCRiSPs. It should be relatively straightforward to extend the findings of these studies to other svCRiSPs and in the process gain additional insight into the mechanisms of action of the toxin and its role in the complex physiological mechanisms involved in snakebite envenomation.

Author Contributions: M.S., E.E.S., and P.D. designed the experiments. M.S., O.S., A.R., A.C., J.A.G., E.S.S., and E.S. conducted the experiments. M.S., A.R., J.S.O., and J.A.G. performed the bioinformatics analysis. M.S. analyzed the data and interpreted the data. M.S., J.A.G., P.D., and E.S.S. wrote and reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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